For Reference

NOT TO BE TAKEN FROM THIS ROOM

For Reference

NOT TO BE TAKEN FROM THIS ROOM

Ex libris
universitatis
albertaensis

UNIVERSITY OF ALBERTA
QUAECUMQUE VERA

Digitized by the Internet Archive
in 2019 with funding from
University of Alberta Libraries

https://archive.org/details/Taylor1966

THE UNIVERSITY OF ALBERTA

THE EFFECT OF SOME CATIONS ON THE RELEASE OF NORADRENALINE FROM RAT HEARTS

by

GRAHAME S. TAYLOR

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

1966

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Effect of some Cations on the Release of Noradrenaline from Rat Hearts" submitted by Grahame S. Taylor in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Isolated perfused rat hearts released tritiated noradrenaline (^{3}H-NA) from previously labelled storage sites when perfused with normal Krebs-bicarbonate solution. Omission of calcium or the addition of different amounts of Na_2EDTA(disodiumethylenediaminetetraacetate) to a calcium free medium, elevated the efflux of ^{3}H-NA above control levels obtained by perfusion with normal Krebs-bicarbonate solution. The increased efflux caused by perfusion with calcium free, or calcium free + Na_2EDTA media, could be reversed by the addition of calcium and removal of Na_2EDTA. The chelating agent Na_2EDTA was observed to possess a stimulating action on release of ^{3}H-NA.

Stimulation of ^{3}H-NA release above control levels was demonstrated with excess potassium and with noradrenaline (NA). The stimulating action of the latter two agents was reduced when a calcium free medium perfused the tissue before and during stimulation. Stimulation by excess potassium and NA was effective when hearts were perfused both before and during stimulation with media in which calcium was replaced by strontium. Both a low sodium and a high magnesium concentration in the Krebs media caused an elevation of the efflux of ^{3}H-NA. Substitution of barium for calcium caused a sharp increase in efflux.

Acetylcholine caused a small and approximately equal increase in the rate of release of ^{3}H-NA in both normal and atropine treated

hearts, although in the former it slowed the heart rate while in the latter it increased the rate.

ACKNOWLEDGEMENTS

For guidance, encouragement and constructive criticism during the progress of this work, I express sincere gratitude to my Supervisor, Dr. C.W. Nash.

To my wife, Judith, I wish to express my appreciation for her patience during the typing of this thesis.

I wish also to acknowledge with thanks the financial support of the Alberta Heart Foundation.

To My Parents

TABLE OF CONTENTS

Page

INTRODUCTION 1

I LITERATURE REVIEW 3

1(a) Historical

1(b) Adrenergic innervation of heart

1(c) Characteristics of subcellular distribution of noradrenaline

1(d) Uptake of noradrenaline

1(e) Cations and release of catecholamines

II METHODS 16

II(a) Heart perfusion and labelling

II(b) Perfusion media

II(c) Counting techniques

III RESULTS 20

IV DISCUSSION 44

V SUMMARY AND CONCLUSIONS 58

BIBLIOGRAPHY 60

APPENDIX I 69

APPENDIX II 73

INTRODUCTION

Several investigators (Douglas and Rubin, 1961b, Douglas and Poisner, 1962, Boullin, 1965) have demonstrated the involvement of various cations (Na^+, Ca^{++}, Mg^{++}) in the release of neurohumors and endocrine secretions but the intimate part played by these cations in the release mechanism is as yet unknown. Douglas and Rubin have postulated that the entry of calcium ion into the chromaffin cell is a prime requisite for the secretion of catecholamines from the cat adrenal glands in response to various secretogogues including acetylcholine and depolarising concentrations of potassium ions. As several similarities exist between the vesicles obtained from sympathetic nerve endings (Potter 1966) and the catecholamine storage granules of adrenal chromaffin tissue (Hagen 1959) it was of interest to determine if the release of noradrenaline (NA) from a sympathetically innervated structure was also influenced by the extracellular ionic environment.

Burn and Gibbons (1964) provided the first indirect evidence that calcium ions were essential at some site on the adrenergic neuronal membrane for nerve impulses to release the sympathetic transmitter. With the aid of tritium labelled catecholamines of relatively high specific activity Boullin and Brodie (1965) demonstrated that changes in the ionic concentration of the perfusion medium in the isolated cat colon altered the efflux of tritiated amine induced by stimulation of the postganglionic nerves. In the

present study the endogenous store of catecholamines in isolated rat hearts were labelled with tritiated noradrenaline (^{3}H-NA) and the efflux of the tritium was followed when the hearts were perfused with various isotonic solutions with different ionic compositions. In addition, in view of the reports (Richardson and Woods 1959, Angelakos and Bloomquist 1965) that increases in inotropic and chronotropic actions which result from administration of acetylcholine to atropinised isolated heart preparations (i.e. nicotinic effects) are mediated through the release of NA, a study of the influence of acetylcholine on the tritium efflux curves from ^{3}H-NA labelled hearts was carried out.

1 LITERATURE REVIEW

1 (a) Historical

One hundred years have now passed since the observation by Vulpian that watery extracts of adrenal glands of many species contained a substance which gave a rose-carmine colour when exposed to air. Some 50 years later one of the catecholamines responsible for this colour reaction was isolated and subsequently synthesised. In 1904 Stolz synthesised NA and made the first observations on its biological properties noting, that it was as active as adrenaline (A) in raising the blood pressure of animals, but less toxic.

During the period between 1904 and 1948, when NA was shown by von Euler to be present in and the specific transmitter substance of adrenergic nerves numerous investigators reported evidence for a "special and unknown" factor which exerted remote sympathomimetic actions but which was different from A (Loewi 1921, Cannon and Uridil 1921, Cannon and Rosenblueth 1933). By the use of more precise preparative and analytical methods differences between the active organ substances and A were detected and agreement was found with NA (von Euler 1948). In confirmation of these observations subsequent authors noted that the activity in most organ extracts from sympathetically innervated structures was in all probability chiefly due to NA (Bacq 1947, Schmiterlöw 1948).

Having established the important role of NA the next problem concerned the location and binding of the transmitter substance. A comparison of the amounts of NA in the splenic nerve and the spleen indicated that the transmitter must be accumulated in the nerve terminals. By using the homogenisation procedure of Hillarp (1955), von Euler showed that the sediment produced by high speed centrifugation of the primary supernate contained NA, which was biologically inactive prior to treatment with heat or acid, thus providing evidence for structurally bound NA (von Euler and Hillarp 1956). In confirmation of these observations an electronmicrograph taken by von Euler and Bahr in 1956 showed that the high speed sediment from homogenised splenic nerves was rich in osmiophilic granules of about 500-1000Å in diameter (von Euler 1966).

Since 1945 many attempts have been made to apply fluorescence techniques to clarify a number of problems involved in the innervation of tissues supplied with adrenergic nerves. In 1962, through the work of Falck, it finally became possible to demonstrate the finest terminals by means of their content of NA. This was done by applying a fluorescent technique based on interaction of monamines including NA with formaldehyde, a procedure previously studied by Eränkö (1955). The recent disclosure of monaminergic nerve paths within the central nervous system, greatly extended through the work of Fuxe (1965) and others, has opened up new and fruitful fields of the highest significance.

1 (b) Adrenergic innervation of heart

The supply of autonomic nervous system fibres in the various parts of the heart has been studied repeatedly with conventional histological techniques. Previous studies were unable to identify with certainty the adrenergic fibres but it is now possible to do so quite precisely with the fluorescence technique of Hillarp and Co-workers (Falck, 1962).

The adrenergic innervation of the rabbit and guinea pig heart was recently studied by Angelakos et al (1963) who found an excellent correlation between histochemical density of fluorescence and chemical content of NA. Furthermore, fluorescence histochemistry indicated that the catecholamine content of the atria was confined within nerve structures. Dahlstrom et al (1965) reported similar findings for dog heart. In both studies mentioned above it is interesting to note that no catecholamine containing cells (chromaffin cells) of any kind were observed in the heart muscle specimens studied. By a combination of radioautography and electronmicroscopy Wolfe and Potter (1963) investigated the disposition of ^{3}H-NA after infusion of the labelled amine into rats. In rat right atrial tissue electronmicroscopic radioautography localised the grains over non-myelinated axons containing granulated vesicles. Furthermore, after homogenisation and sucrose density gradient techniques the majority of the radioactivity appeared in the granulated vesicle fractions along with endogenous NA.

Of the various procedures available for denervation of the heart, excision and reimplantation of the heart ("orthotopic cardiac autotransplantation") is by far the most effective (Cooper 1965). Three days after that procedure the uptake of dl-^{3}H-NA was reduced to 6.1% of normal. Together with this loss of NA binding capacity, and virtually complete loss of ability to store NA, the indirectly acting sympathomimetic amines lost most of their cardiac effect.

Immunosympathectomised rats (Iversen et al 1966) display an almost complete atrophy of sympathetic nerve cells and studies on the physiologic disposition of ^{3}H-NA in these animals showed that the reduction of the ability to accumulate the labelled amine paralleled the reduction in the endogenous catecholamine content of these tissues. For rat heart, Iversen et al (1966) reported the uptake of ^{3}H-NA to be less than 10% of normal.

The above studies emphasise the importance of the adrenergic innervation of the heart for the normal accumulation and binding of NA.

1 (c) Characteristics of subcellular distribution of Noradrenaline

The existence of specific intracellular storage granules for catecholamines was first demonstrated for adrenal medullary tissue in 1953 (Blaschko and Welch 1953; Hillarp et al 1953). Using similar centrifugation techniques von Euler and Hillarp (1956) studied the storage of NA in the splenic nerves and spleen of oxen. They demonstrated that some of the NA stored in this tissue could be isolated from homogenates containing subcellular particles.

Electron microscopic studies on catecholamine deposits were initially done on cells of the adrenal medulla,and special membrane-bound granules or vesicles, which intensely reduced osmium tetroxide, were recognised (Lever 1955). In adrenergic axons and endings innervating the pineal gland and in the splenic nerve, a plurivesicular material was described by De Robertis and Pellegrino de Iraldi (1961, 1961a). This consisted of clear homogenous vesicles intermingled with granulated vesicles containing a dense granule of reduced osmium. Similar components have been observed in various parts of the sympathetic nervous system (Grillo and Palay 1962; Richardson 1962).

Potter and Axelrod (1962) found that particles obtained from rat hearts, containing endogenous and exongenous (tritium-labelled) NA, sedimented during gradient centrifugation with particles of "microsomal" size. Similar results were obtained for endogenous and ^{3}H-NA in the salivary gland and vas deferens, and for ^{3}H-NA in the pineal and adrenal

glands (Potter and Axelrod 1963). Subsequent modifications of methodology have yielded isolated NA containing granules, relatively uncontaminated by ribonucleoprotein and glycogen granules (Potter 1966). The physical properties of the splenic nerve vesicles which contain NA (von Euler and Lishajko 1961; Stjärnë 1964) and of the rat heart vesicles which retain endogenous and labelled NA (Potter and Axelrod 1963a) have been well studied and are very similar. Both are lysed by detergents, but are moderately resistant to lysis by suspension in water, or by freezing and thawing. Both rapidly lose NA in warm solutions and both are able to retain NA against large gradients.

As yet, no preparation of vesicles containing NA has been obtained in a sufficiently pure form to permit accurate measurements of the concentration of lipids, proteins, adenine nucleotides and other constituents of the vesicles, comparable to the measurements in the isolated adrenal chromaffin granules by Hillarp (1958). The average content of NA per granulated vesicle is not known. However, the presence or absence of several ensymes in partially purified preparations of heart vesicles and the presence of ATP in both splenic nerve and heart vesicles has been demonstrated (von Euler 1956; Potter and Axelrod 1963a).

The molar ratio of NA: ATP has been found to be about 4:1 for a variety of nerve granule preparations (Stjärnë 1964). In view of the consistency of this ratio and the observation that the ATP is moderately

inert within the granules, ATP has often been suggested as an ionic complexing agent responsible for concentrating amines within vesicles or as the final binding substance, or both. Weak catecholamine-ATP complexes have been demonstrated in solutions (Weiner and Jardetzky 1964). Magnesium ion activated ATPases are present in chromaffin granules (Banks and Blaschko 1966) and both magnesium and calcium activated ATPases occur in the best present preparations of heart vesicles which store NA. Since secretion of catecholamines from the adrenal medulla is calcium dependent Potter (1966) speculates that stimulus secretion coupling may involve an ion activated enzyme, like ATPase, which either causes an abrupt disruption of a catecholamine binding complex or causes a sudden change in membrane permeability.

Of the NA contained within sympathetic nerve endings in rat heart, no more than 77% has been isolated in small particles (Potter and Axelrod 1963). This raises the question of the function and relation of the soluble NA to vesicle-bound NA. Prelonged homogenisation permits better cellular disruption but also decreases the yield of amine containing particles (Iversen and Whitby 1963). Repeated sedimentation and resuspension of particles is reported to have a similar effect. For this reason it has been suggested that much of the NA appearing in soluble form is released from storage vesicles during the process of cell disruption (Potter and Axelrod 1963). On the other hand some NA must normally be present in the cytoplasm

in equilibrium with that in the vesicles. von Euler and Lishajko (1962) have suggested that this soluble form is important as the source of transmitter released by nerve stimulation.

In view of the relative stability of isolated nerve granules to a number of changes in the environment it appears unlikely that changes in the intraneuronal milieu accompanying the depolarisation of the axonal membrane could be solely responsible for release of NA. The only structure which is definitely known to be profoundly changed by a propagated nerve impulse is the axonal membrane. Stjärne (1966) has recently postulated that NA lipid-bound in the axonal membrane itself, or possibly contained in some "membrane-bound vesiculated structure" might represent the small NA pool from which the neurotransmitter release occurs on depolarisation of the axonal membrane.

1 (d) Uptake of Noradrenaline

The uptake of NA into the tissue stores of sympathetically innervated organs has been demonstrated in many ways; as an increase over endogenous levels following injection of levo-NA (Campos and Shideman 1962), as the uptake of labelled amine after injection of dl-^{3}H-NA (Hertting and Axelrod 1965; Crout 1964), or as the difference between arterial and venous levels in the blood perfusing isolated organs (Gillespie and Kirpekar 1963).

It is now generally considered by some workers that in the sympathetic nervous system re-entry into the nerve ending and not enzymatic destruction is the main mechanism for terminating the biological action of circulating or locally released NA (Rossell, Kopin and Axelrod 1963). The potentiating effects of many drugs with regard to sympathetic function may be visualised as an inhibition or prolongation of the uptake mechanism (Dengler et al 1961).

Relatively little is known of the detailed properties of the catecholamine uptake process. Dengler et al (1962a) have studied *in vitro* the uptake of dl-^{3}H-NA by slices of cerebral cortex, heart and spleen and by isolated pineal bodies of the cat (Dengler et al 1962b). These workers found the uptake obeyed saturation kinetics of the Michaelis Menten type and required metabolic energy. The carrier mechanism became saturated at levels of 100 mμgm per ml and was accompanied by a

simple diffusion, the rate of which was proportional to concentration at all levels. Crout (1964) has shown that guinea pig hearts *in vitro* concentrate ^{3}H-NA to levels 8 to 12 times those in blood within a minute after injection. Saturation of this mechanism at blood levels of the order of 1 μM was taken to be in accord with a carrier mechanism for uptake. When rat hearts were perfused with NA concentrations exceeding those necessary to saturate the transport phenomenon, an unusual uptake phenomenon was observed by Iversen (1965a). At critical concentrations of NA (about l μg/ml for NA) a striking increase in the uptake of catecholamines appeared. This new uptake phenomenon was designated Uptake$_2$ by Iversen (1965a). Uptake$_2$ did not distinguish between optical isomers of NA as did Uptake$_1$ which is more specific for 1-NA. It was saturable only at concentrations of 85 μg/ml NA. Since Uptake$_2$ emerges only at toxic levels of NA it is not usually a factor in studies of uptake from the circulation *in vivo*.

A great number of drugs can inhibit NA uptake in slices (Dengler 1965, Dengler 1961), in perfused organs (Iversen 1965,b,c,), and *in vivo* (Hertting et al 1961), as well as in subcellular particles (von Euler and Lishajko 1965). Oubain was of particular interest since it was without effect on granules but markedly inhibited uptake in slices. Potassium which is known to antagonise the effects of cardiac glycosides on cation transport (Glynn 1957) can also counteract the effects of these steroids on NA uptake in rabbit heart slices. These findings have led Titus and Dengler (1966) to indicate that the uptake of NA may be linked with a cation transport mechanism.

1 (e) Cations and Release of Catecholamines

In 1961 Douglas and Rubin investigated the actions of acetylcholine as a medullary secretogogue during perfusion of the cat's adrenal gland with media of differing ionic composition. The experiments showed that acetylcholine still caused secretion of catecholamines when all sodium and potassium was omitted from the perfusion medium (tonicity being maintained with sucrose). By contrast, the response to acetylcholine was profoundly depressed when calcium was omitted from the perfusion medium, and over a wide range of calcium concentrations the response to acetylcholine increased with increasing calcium concentration so that at 17.6 mM the output of catecholamines to the test dose of acetylcholine was doubled. In addition evidence was obtained that calcium itself could evoke catecholamine secretion. These findings coupled with the known ability of acetylcholine to increase the permeability of membranes at other sites in the body, led Douglas and Rubin (1961b) to propose that acetylcholine probably stimulated the chromaffin cell to secrete by promoting an increased uptake or influx of calcium ions. Further support for this hypothesis was provided by Douglas and Poisner (1962) when they demonstrated that acetylcholine increased calcium-45 uptake in the adrenal medulla. Excess potassium was shown by Vogt (1959) to evoke catecholamine secretion by a direct action on the chromaffin cell. That this action of potassium required calcium in the extracellular environment

was shown by Douglas and Rubin (1961), who also showed that the intensity of the secretory response to excess potassium varied with the extracellular calcium concentration over a wide range and that excess potassium greatly increased the uptake of calcium-45 by the adrenal medulla (Douglas and Poisner 1962).

A rather different piece of evidence to support the view that calcium entry is a critical link in "stimulus-secretion coupling" has come from experiments in which calcium itself in the absence of any of the familiar secretogogues evokes catecholamine secretion. Although calcium itself has been shown to have little effect on the adrenal medulla in normal circumstances, after a period of perfusion with a calcium free medium reintroduction of this ion induces secretion of catecholamines. Douglas (1965) proposed that the stimulant action of calcium is due to the penetration of the plasma membrane of the chromaffin cell by this ion.

The stimulant effect of barium on catecholamine release observed by Douglas and Rubin (1964) was somewhat different than that of calcium. Barium was demonstrated to act in its own right and not merely by freeing calcium from sites of binding; it was fully effective in media free of calcium and containing ethylenediaminetetraacetic acid (EDTA). Magnesium was quite without stimulant effect on cats adrenal glands, its action, on the contrary, was strongly inhibitory. It opposed not only the stimulant effect of calcium and barium, but also those of acetylcholine and

potassium.

In contrast to the considerable evidence that the concentration of extracellular cations exerts a profound effect on the release of catecholamines from the adrenal medulla the role of the external ionic environment in the release of NA from sympathetic nerves has been neglected.

Burn and Gibbons(1964) provided indirect evidence that calcium was essential at some site on the adrenergic neuronal membrane for nerve impulses to release the sympathetic transmitter. Using ^{3}H-NA, Boullin (1965) and Boullin and Brodie (1965) were able to confirm the results of Burn (1964) that calcium was necessary for the stimulated release of ^{3}H-NA from the cat colon. These workers further showed that normal responses to nerve stimulation were restored by replacement of calcium with barium but not magnesium. Following perfusion with calcium free solutions, replacement of calcium or substitution of barium evoked a spontaneous release of ^{3}H-NA. These results are qualitatively similar to those found for the isolated adrenal medulla by Douglas and Co-workers.

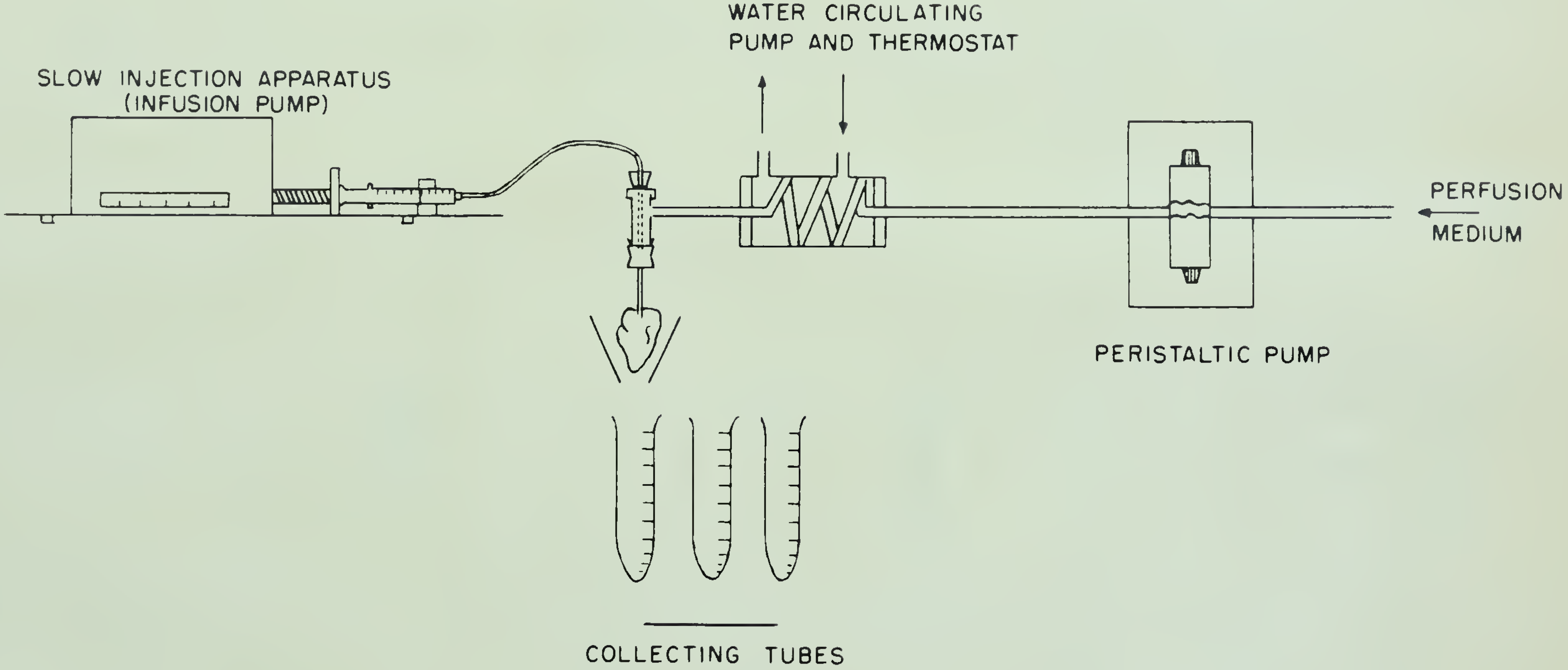
II METHODS

II (a) Heart Perfusion and Labelling

Sprague-Dawley male rats weighing between 100-150 g were killed by a blow on the head; the hearts were excised, cannulated at the aortic arch with a dulled 18 guage needle and perfused at 3 ml per minute by a constant flow modification of the Langendorff method. Krebs-bicarbonate perfusion medium, aerated with carbogen (95% 0_2, 5% CO_2) and warmed to 38°C by passing through a heating coil was delivered to the hearts by a peristaltic perfusion pump (Harvard Instrument Company), and the perfusate was collected in a series of 50 ml graduated centrifuge tubes which were changed at 3 minute intervals. The endogenous NA stores of the heart were labelled by slow infusion of tritiated dl-noradrenaline (S.A.4.7 C/mM-New England Cor.) into the perfusion medium at a point just before entering the hearts and at a rate sufficient to give a concentration of 5.5 ng/ml/min. (Appendix I). After 9 minutes the infusion of radioactive amine was terminated and the efflux of the labelled amine followed by collecting the perfusate from the hearts for a further 78 minutes. Test drugs were similarly infused into the perfusion medium by means of the Palmer slow injection pump and the ionic composition of the perfusion medium was altered according to the particular experimental schedule. Apparatus for the simultaneous perfusion of 4 isolated rat hearts is

FIG. 1

APPARATUS



illustrated in Fig. 1.

II (b) Perfusion Media

The ionic compositions of the perfusion media employed are listed in Appendix I. All hearts were initially perfused with normal Krebs-bicarbonate solution, but during tests of the influence of altered ionic environment on the rate of release of ^{3}H-NA, the heats were perfused with a variety of modified Krebs solutions. For example, to test the influence of calcium on the release of NA the hearts were perfused with a modified Krebs solution in which the calcium chloride was omitted and replaced by strontium chloride or barium chloride, and to some of which varying amounts of Na_2EDTA (disodiumethylenediaminetetraacetate) or EGTA (ethylene bisglycol (-aminoethylether) tetraacetic acid), were added. When excess potassium was employed as a depolarising agent sufficient potassium chloride was substituted for sodium chloride in the Krebs solution to give a potassium ion concentration of 59 mM. Similar substitutions were made with sucrose or sodium chloride when the influence of other ions were tested.

II (c) Counting techniques

A 1 ml aliquot of the total efflux collected every 3 minutes (about 9 ml) was transferred to 10 ml of scintillation fluid (Appendix I) and the radioactivity recorded by means of a Nuclear-Chicago 725

scintillation spectrometer. After checking the counter by the reproductibility of a tritium standard, the samples were each counted for a 4 minute period. The counting efficiency was determined and found to be approximately 10%.

At the end of the prescribed perfusion period the hearts were immediately removed from the perfusion apparatus, sliced about a longitudinal axis through the apex, blotted dry and frozen with liquid nitrogen. With the aid of a stainless steel die and punch (Callingham and Cass 1963), the hearts were pulverised and tableted. The tablet of pulverised frozen tissue was homogenised with a Potter Homogeniser in 10 ml extration medium (ice cold salt saturated n-butanol - Appendix I) and shaken to extract the NA (von Euler et al 1951). The butanol extract was separated from the tissue by centrifugation at 2000 rpm after which the NA extract from the tissue was separated by throwing it out of the butanol extract into 0.01N hydrochloric acid by the addition of 15 ml of heptane and shaking. The concentration of NA located in the lower aqueous phase (0.01N HCl) was determined by the trihydroxyindole fluorescent assay procedure using an Aminco-Bowman spectrophotofluorometer at an activation wavelength of 385 mμ, fluorescence wavelength 485 mμ. A 0.5 ml aliquot of the acid extract, to which was added an equal value of Krebs solution, was counted for its tritium content in 10 ml of scintillation fluid and, after the volume correction, the figure obtained was taken as the counts per minute from ^{3}H-NA left in the heart at the

end of the perfusion period.

III RESULTS

III (a) Perfusion with Normal Krebs

The efflux of tritiated amine from the isolated rat hearts is shown in Fig. 2. The perfusion medium was normal Krebs bicarbonate solution and each point represents the average counts per minute released over a three minute period expressed as a percentage of the total counts per minute present in the hearts at the beginning of the release period. The counts per minute present in the hearts at the beginning of the release period are obtained by summation of the efflux and adding the total counts per minute in the hearts at the end of the perfusion period, as indicated in Appendix II, Table 1.

III (b) Perfusion with Calcium free Krebs

Removal of calcium from the perfusion medium produced an increase over the control efflux with normal Krebs throughout the perfusion period. Addition of 0.2 mM Na_2EDTA to the calcium free perfusion medium did not elevate the level of release above that produced by perfusion with calcium free medium alone but, as indicated in Fig. 3, addition of 2 mM Na_2EDTA to the calcium free medium produced a much higher release of tritiated amine.

III (c) The Releasing Effect of Na_2EDTA

To determine whether the rise in efflux of tritiated amine in the preceding experiments was due to the presence of Na_2EDTA *per se* or

to calcium lack, an experiment was carried out replacing calcium free Krebs + 2 mM Na_2EDTA with calcium free Krebs instead of with normal Krebs containing calcium (2.5 mM) as was done formerly. Fig. 4 illustrates the results of experiments in which 4 hearts were perfused with normal Krebs for 27 minutes. When the perfusion fluid was changed to calcium free Krebs + 2 mM Na_2EDTA and perfusion continued for a further 24 minutes a sharp rise in efflux was observed. The efflux contained at this elevated rate in 2 hearts perfused with the calcium free medium containing 2 mM Na_2EDTA. However, the remaining 2 hearts were perfused with a calcium free medium containing no Na_2EDTA and a decrease in release was evident. It thus appears that Na_2EDTA has some releasing action of its own which is quite reversible when the chelating agent is washed out of the tissue.

III (d) Calcium free + 2 mM Na_2EDTA and Normal Krebs Crossover Experiments

Fig. 5 shows the results of the latter half of a crossover experiment involving normal Krebs and calcium free Krebs + 2mM Na_2EDTA. The 4 separate treatments are designated by hearts A, B, C and D respectively. The hearts designated by letter A (▲) were perfused before the test period with calcium free Krebs + 2 mM Na_2EDTA. During the test period the hearts were perfused with normal Krebs thus replacing the calcium and removing the Na_2EDTA and a decreased efflux occurred. A subsequent perfusion period with calcium free Krebs + 2 mM Na_2EDTA caused the efflux rate to rise again. The hearts designated by letter B (△) were perfused with

calcium free Krebs + 2 mM Na_2EDTA throughout the entire schedule, the release was observed to be elevated above that produced by perfusion with normal Krebs indicated by hearts D (○). If calcium was removed and 2 mM Na_2EDTA added to the perfusion medium after perfusion with normal Krebs, the release of tritiated amine increased and then subsequently decreased when normal Krebs was again restored (hearts C (●)).

III (e) Extended Crossover Experiment

In experiments already described involving perfusion with calcium free Krebs and normal Krebs data was obtained from several hearts and it was therefore of interest to validate these results by consecutive perfusion of one or more hearts with either of the above mentioned media. Fig. 6 shows the results obtained with such an experiment. Hearts A (■) were perfused initially with calcium free medium. After 30 minutes this medium was replaced with Krebs containing the normal amount of calcium. After 15 minutes the calcium free medium was reintroduced and subsequently 2 mM Na_2EDTA was added. Finally normal Krebs was perfused for a further 15 minutes to conclude the experiment. Hearts B (▲) after 30 minutes perfusion with normal Krebs were switched to calcium free medium for 15 minutes and then returned to normal Krebs for a further 30 minutes. Finally, calcium free Krebs + 2 mM Na_2EDTA was employed as the perfusion medium for the last 15 minutes. The results indicate that the release caused by perfusion with calcium free Krebs can be reduced by perfusion with normal Krebs. Furthermore, the releasing action of perfusion with calcium

free Krebs + 2 mM Na_2EDTA can be reversed by the restoration of a normal Krebs medium.

III (f) Perfusion with Strontium substituted Krebs

A strontium substituted Krebs bicarbonate medium was prepared in which calcium was replaced by strontium (2.5 mM) and this medium was employed during the test period after prior perfusion with a calcium free medium containing 2 mM Na_2EDTA as indicated in Fig. 7. The strontium substituted Krebs medium caused a fall in the efflux as compared to hearts perfused with calcium free Krebs + 2 mM Na_2EDTA. When the strontium medium was replaced with the calcium free medium containing Na_2EDTA the efflux rose rather slowly towards its previous elevated level.

III (g) Perfusion with Barium substituted Krebs

When barium at concentrations of 2.5 mM and 5 mM was perfused in calcium free media it invoked release of tritiated amine during test periods as shown in Figs. 8 and 9. The release produced by 5 mM barium in a calcium free medium was considerably larger than that produced by 2.5 mM barium.

III (h) Potassium stimulation of Release

For the test period of this series of experiments a high concentration of potassium was incorporated into the Krebs bicarbonate medium. Hearts perfused with a high concentration of potassium (59 mM)

responded to the stimulus by an increase in efflux as shown in Fig. 10. Removal of this stimulating concentration of potassium and restoration of normal Krebs medium subsequently decreased the efflux. If calcium was omitted from the perfusion medium the stimulating action of potassium was no longer evident. Perfusion with calcium free Krebs evoked a release which was above that produced by normal Krebs.

III (j) Strontium substituted Krebs and stimulation by Potassium

As in the previous experiment a high concentration of potassium (59 mM) was employed as a stimulating agent. When perfused in a strontium substituted medium high potassium was able to stimulate release of tritiated amine. Restoration of strontium substituted Krebs containing normal potassium concentration (5.9 mM) reduced the efflux considerably. Control hearts perfused with a calcium free medium exhibited no such stimulating action when exposed to a high potassium concentration in a calcium free medium (Fig. 11).

III (k) Perfusion with calcium free Krebs and the stimulating action of Noradrenaline

NA at concentrations of 1 μM and 2 μM was introduced into the perfusion medium via the infusion pump during perfusion with either calcium free Krebs or normal Krebs containing 2.5 mM calcium. As shown in Fig. 12 both concentrations of NA invoked release of tritiated amine but hearts perfused with calcium free medium exhibited less release than those

on normal Krebs medium.

III (l) Perfusion with calcium free Krebs + 2 mM Na_2EDTA and the stimulating action of Noradrenaline

The addition of 2 mM Na_2EDTA to the calcium free perfusion medium caused a sustained release of tritiated amine as shown in previous experiments (Fig. 3) and again indicated on the efflux shown in Fig. 13. Infusion of NA at concentrations of 1 μm and 2 μM respectively stimulated an increased rate of efflux in hearts perfused with media containing a normal calcium concentration (2.5 mM), but identical concentrations of NA infused into hearts perfused with calcium free Krebs containing 2 mM Na_2EDTA produced no increase in the rate of efflux of tritiated amine. It thus appeared that the omission of calcium and the addition of Na_2EDTA to the perfusing medium inhibited the stimulating action of NA on ^{3}H-NA efflux.

III (m) Strontium substituted Krebs and Noradrenaline stimulation

The stimulant action of 1 μM and 2 μM of NA is shown in Fig. 14 during perfusion with either strontium substituted Krebs or calcium free Krebs. The stimulant action of NA was evident in hearts receiving the strontium substituted Krebs throughout the duration of the experiment. After perfusion with calcium free Krebs 2 further hearts did not respond to the stimulating agent with an equivalent release of tritiated amine.

III (n) Perfusion with Low Sodium Krebs

Following a perfusion period with normal Krebs a test period

of low sodium Krebs, in which sodium chloride was replaced by an osmotically equivalent amount of sucrose, caused an increased release of tritiated amine above the control rate of release. The increased efflux decreased on returning to a perfusion medium in which the sodium concentration was restored to normal values, as shown in Fig. 15.

III (o) Perfusion with Excess Magnesium

The effects of an excess of magnesium ions are illustrated in Fig. 16. A test period of perfusion with 80 mM magnesium resulted in an increase in release as compared to normal Krebs perfusion. The curve is qualitatively similar to the one obtained using a test period of low sodium Krebs (Fig. 15). The high magnesium concentration was achieved by substitution for sodium.

III (p) The Releasing Action of Acetylcholine

The releasing action of acetylcholine at concentrations of 50 µg/ml and 100 µg/ml in atropinised and non-atropinised hearts is illustrated in Fig. 17. Atropine at a concentration of 10 µg/ml was continuously perfused through 2 hearts. In both atropinised and non-atropinised hearts the concentrations of acetylcholine employed induced a small release of tritiated amine. In the atropinised preparations an increase in heart rate was observed upon administration of acetylcholine. In contrast the non-atropinised preparations ceased to exhibit rhythmic contractions during

the acetylcholine infusion but began to beat again after the acetylcholine was withdrawn. The results indicate that a similar quantity of tritiated amine was released in both preparations and hence exclude the possibility that an increase in rate of beating of the isolated hearts will produce a greater efflux. A further complication of these experiments concerns the possible presence of sympathetic ganglia within the isolated heart preparation. However, the results of further experiments in which nicotine was used as the stimulating agent were equivicol and failed to provide conclusive evidence for a ganglionic mechanism of release in rat hearts.

FIG. 2

NORMAL KREBS PERFUSION

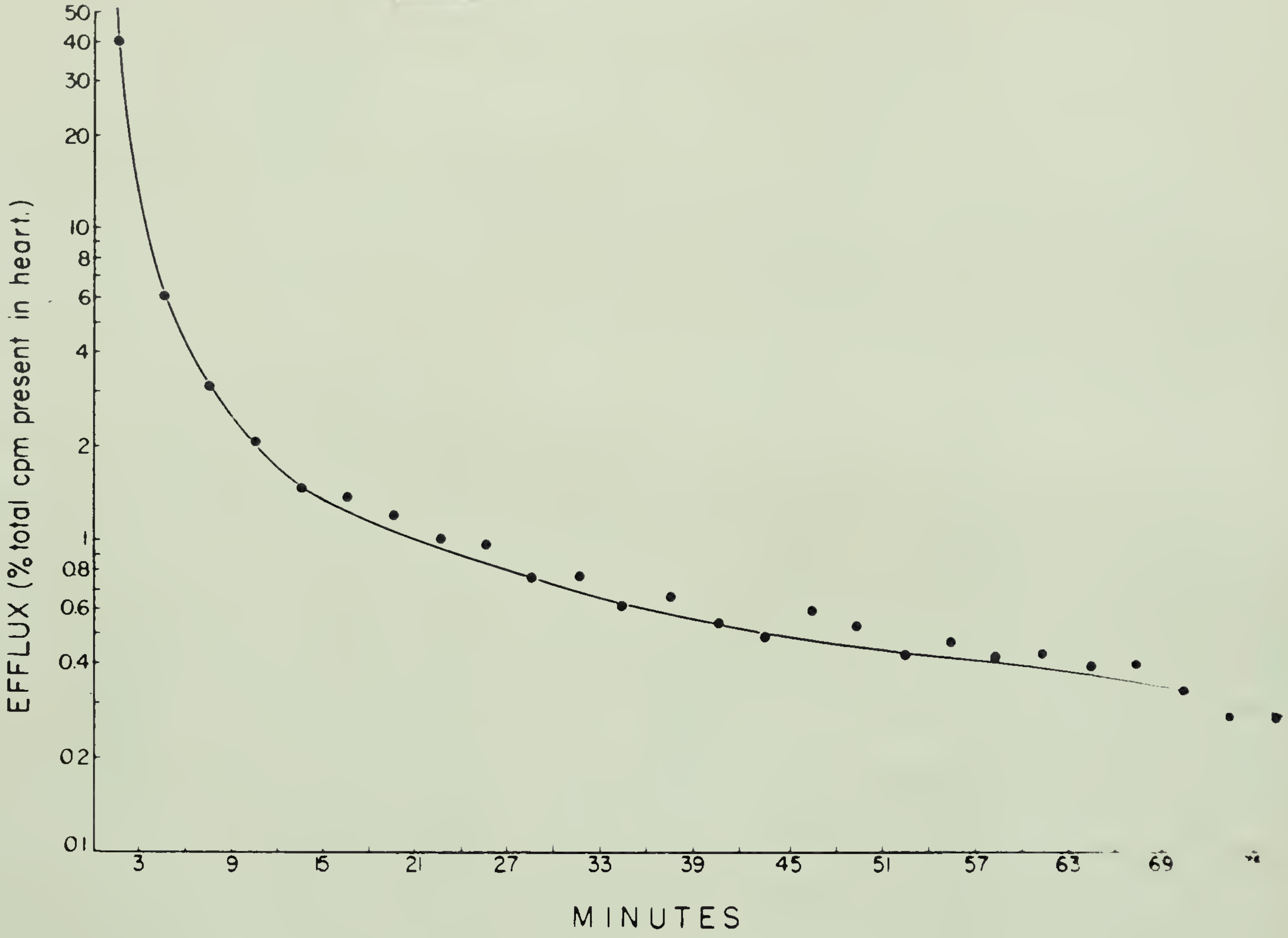


FIGURE 2

Perfusion with Normal Krebs-bicarbonate Solution

The efflux of tritiated amine, expressed as a percentage of the total cpm present in the heart at the beginning of the release period, is plotted against time (minutes). Curves similar to Fig. 2 were obtained from more than 10 isolated hearts perfused with normal Krebs-bicarbonate solution. Combining the results of 2 of these experiments provided Fig. 2 as indicated in Appendix II, Table 1.

FIG. 3

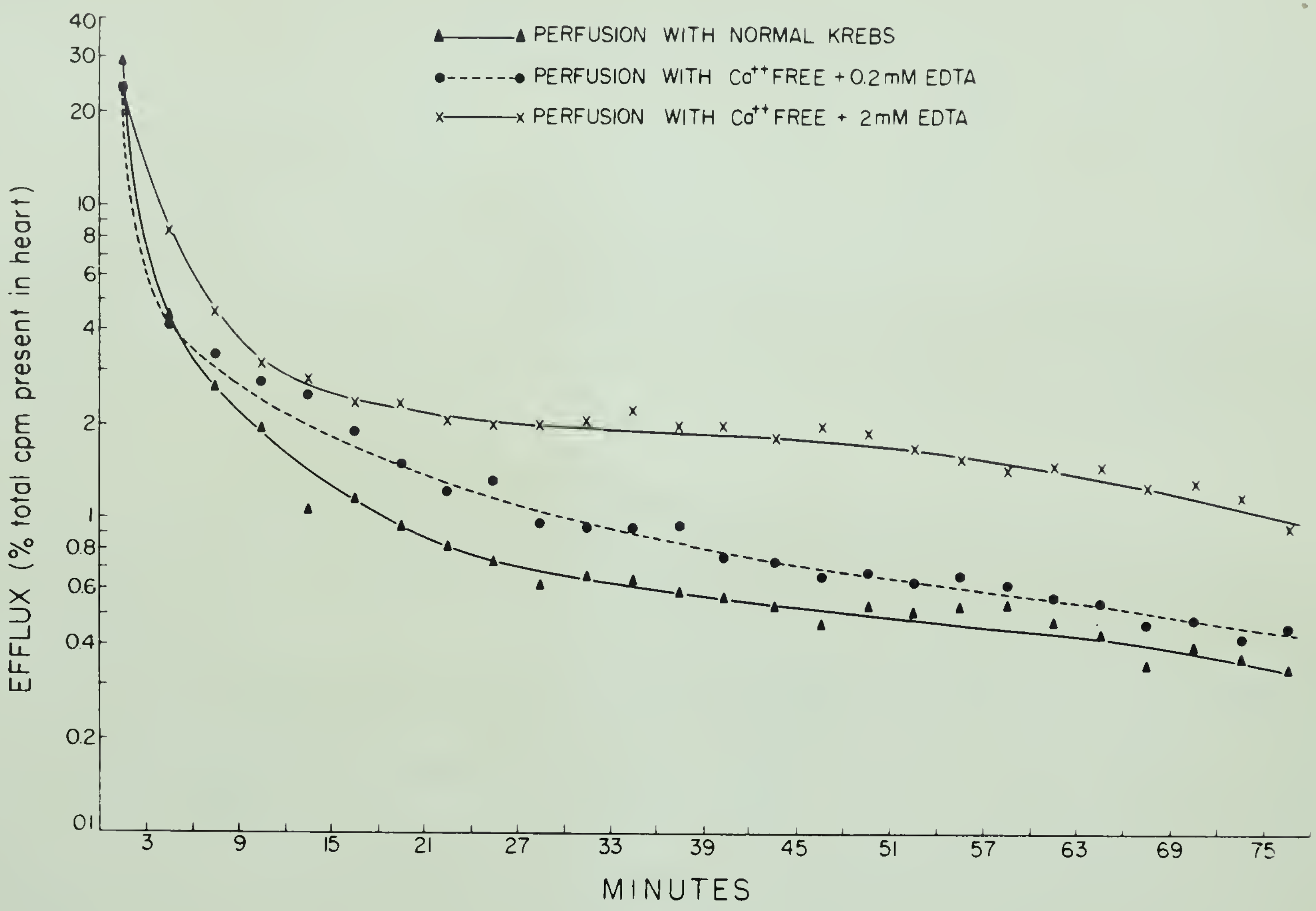

PERFUSION WITH NORMAL KREBS
PERFUSION WITH Ca++ FREE + 0.2 mM EDTA
PERFUSION WITH Ca++ FREE + 2 mM EDTA
EFFLUX (% total cpm present in heart)
40
30
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
01
3
9
15
21
27
33
39
45
51
57
63
69
75
MINUTES

FIGURE 3

Comparison of perfusion with normal Krebs-bicarbonate medium and calcium free media containing 0.2 mM and 2 mM EDTA

Removal of calcium from the perfusion medium elevated the efflux of ^{3}H-NA above control levels obtained with normal Krebs-bicarbonate solution. The addition of 0.2 mM EDTA to a calcium free medium did not increase the efflux above that obtained with calcium free medium alone. The increased efflux caused by perfusion with calcium free medium alone is shown in part in Figs. 10 and 11. Addition of 2 mM EDTA to a calcium free medium caused a sustained release of ^{3}H-NA.

Each point represents the mean of four hearts except in the upper curve (calcium free + 2 mM EDTA) where two hearts were used.

The corresponding data are given in Appendix II, Table 2.

FIG. 4

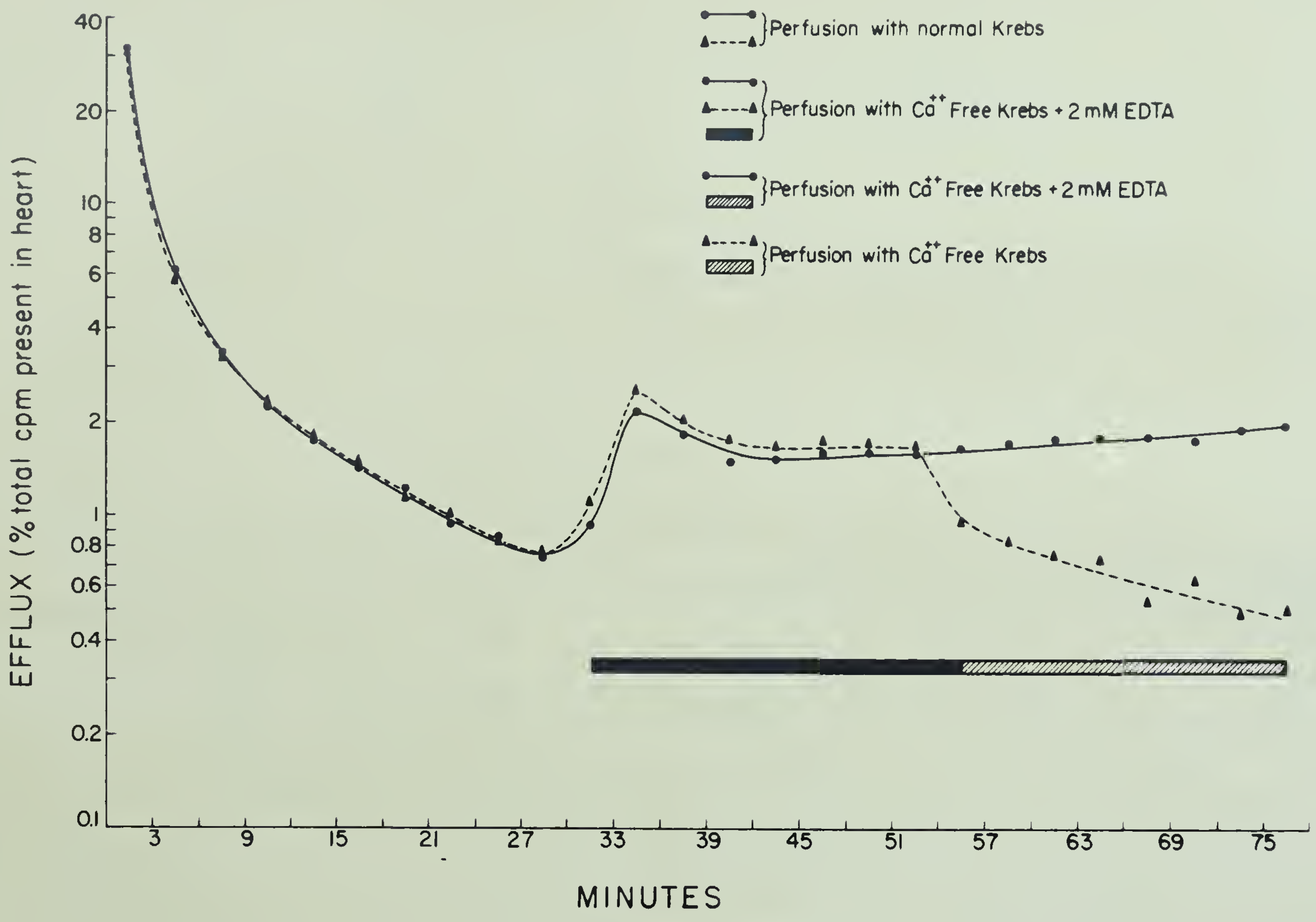

Perfusion with normal Krebs
Perfusion with Ca⁺⁺ Free Krebs + 2 mM EDTA
Perfusion with Ca⁺⁺ Free Krebs + 2 mM EDTA
Perfusion with Ca⁺⁺ Free Krebs
EFFLUX (% total cpm present in heart)
40
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
0.1
3
9
15
21
27
33
39
45
51
57
63
69
75
MINUTES

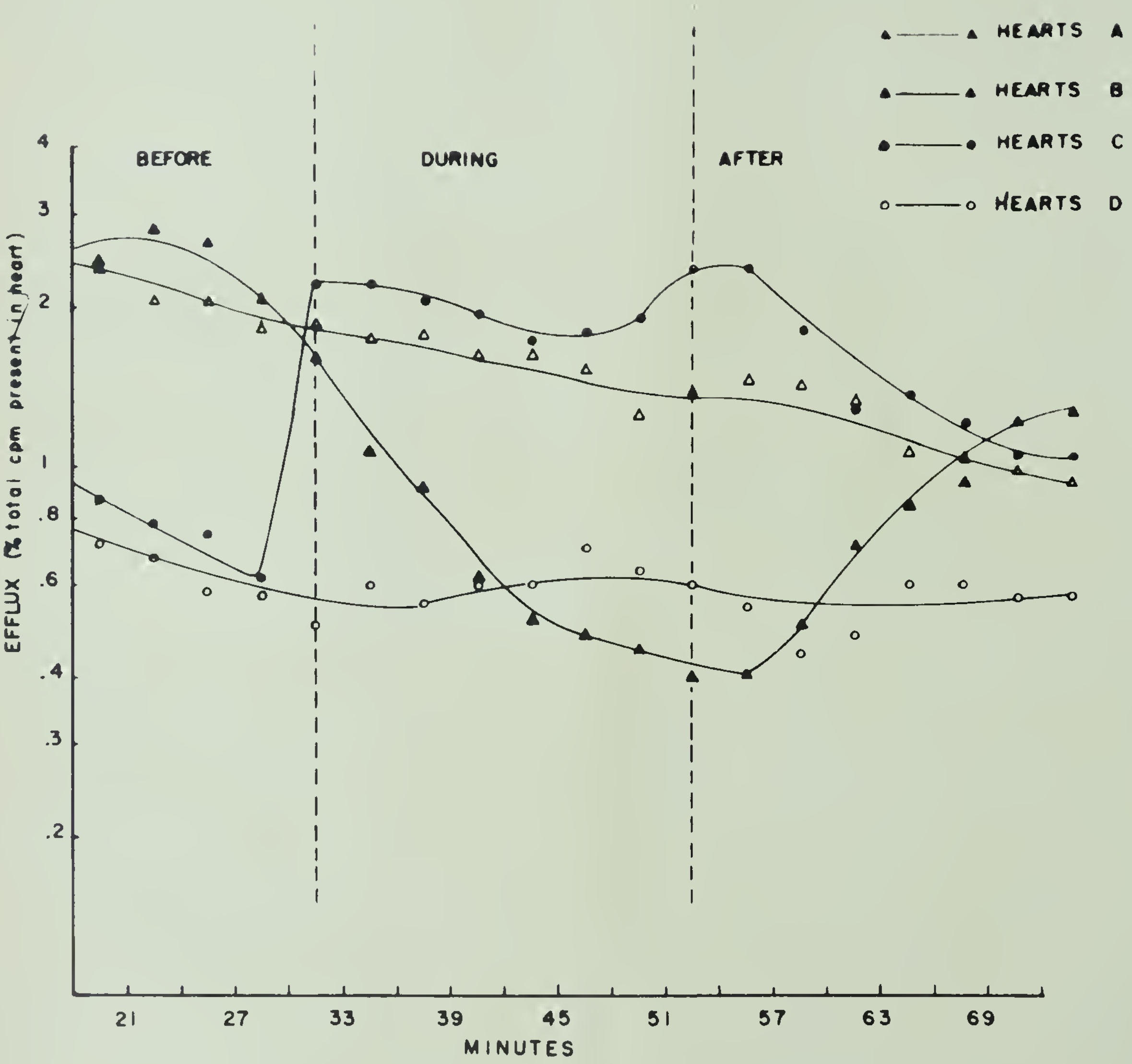
FIGURE 4

The Releasing Effects of EDTA

Four hearts were initially perfused with normal Krebs-bicarbonate solution and then switched to calcium free media containing 2 mM EDTA. The increased efflux caused by addition of EDTA could be reversed by removal of the chelating agent and transferring two of the hearts to a calcium free medium.

The corresponding data are given in Appendix II, Table 3.

FIG 5



HEARTS A
HEARTS B
HEARTS C
HEARTS D
BEFORE
DURING
AFTER
EFFLUX (% total cpm present in heart)
4
3
2
1
.8
.6
.4
.3
.2
21
27
33
39
45
51
57
63
69
MINUTES

FIGURE 5

Crossover experiment with EDTA and Normal Krebs-bicarbonate

Hearts B were perfused throughout with a calcium free medium containing 2 mM EDTA. Hearts A were initially perfused with the same medium but were switched to a normal Krebs-bicarbonate medium during the test period (designated "during"). After the test period the hearts A were then transferred back to a calcium free medium containing 2 mM EDTA. Hearts D were perfused throughout with normal Krebs-bicarbonate solution. Initially hearts C were perfused with normal Krebs-bicarbonate medium and then were switched to a calcium free medium containing 2 mM EDTA. After the test period hearts C were returned to normal Krebs-bicarbonate solution.

The corresponding data are given in Appendix II, Table 4.

FIG. 6

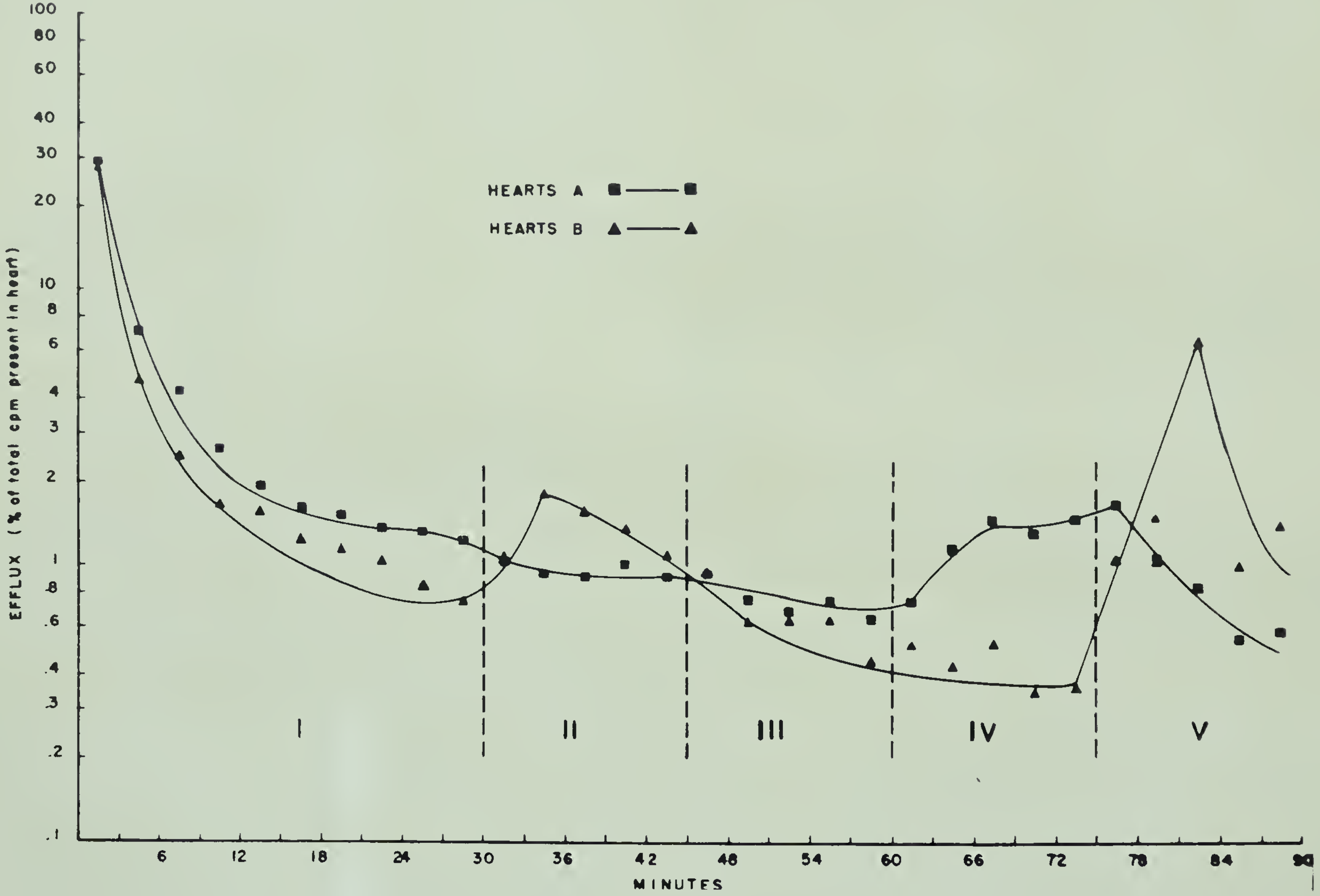

HEARTS A ■——■
HEARTS B ▲——▲
EFFLUX (% of total cpm present in heart)
100
80
60
40
30
20
10
8
6
4
3
2
1
.8
.6
.4
.3
.2
.1
I
II
III
IV
V
6
12
18
24
30
36
42
48
54
60
66
72
78
84
90
MINUTES

FIGURE 6

Extended Crossover Experiment

Hearts A were perfused with the following media during the 5 perfusion periods:-

Period	Perfusion Media
I	Calcium free Krebs-bicarbonate solution.
II	Normal Krebs-bicarbonate solution.
III	Calcium free Krebs-bicarbonate solution.
IV	Calcium free Krebs-bicarbonate solution + 2 mM EDTA.
V	Normal Krebs-bicarbonate solution.

Hearts B were perfused with the following media during the 5 perfusion periods:-

Period	Perfusion Media
I	Normal Krebs-bicarbonate solution.
II	Calcium free Krebs-bicarbonate solution.
III	Normal Krebs-bicarbonate solution.
IV	Normal Krebs-bicarbonate solution.
V	Calcium free Krebs-bicarbonate solution + 2 mM EDTA.

The corresponding data are given in Appendix II, Table 5.

FIG. 7

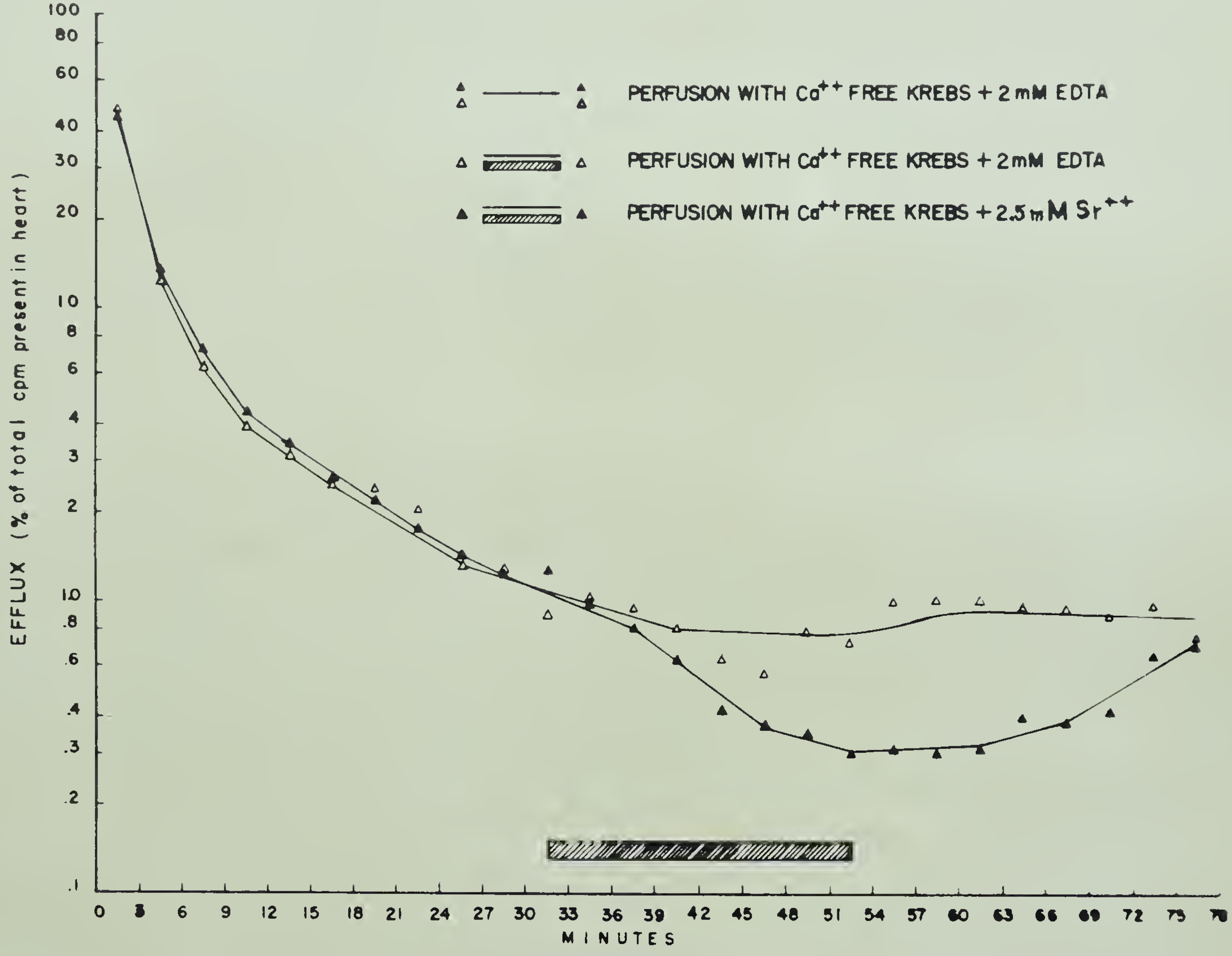

▲ ▲ —— ▲ ▲ PERFUSION WITH Ca^{++} FREE KREBS + 2 mM EDTA
△ ▨ △ PERFUSION WITH Ca^{++} FREE KREBS + 2 mM EDTA
▲ ▨ ▲ PERFUSION WITH Ca^{++} FREE KREBS + 2.5 mM Sr^{++}
EFFLUX (% of total cpm present in heart)
100
80
60
40
30
20
10
8
6
4
3
2
1.0
.8
.6
.4
.3
.2
.1
0
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
51
54
57
60
63
66
69
72
75
78
MINUTES

FIGURE 7

Perfusion with Strontium substituted Krebs solution after calcium free + 2 mM EDTA

After perfusion of four hearts with a calcium free medium containing 2 mM EDTA for 30 minutes, three hearts were switched to a strontium substituted media during the test period and finally replaced on the calcium free media containing 2 mM EDTA. The control heart for the experiment was perfused with calcium free Krebs + 2 mM EDTA throughout the entire schedule.

The corresponding data are given in Appendix II, Table 6.

FIG. 8

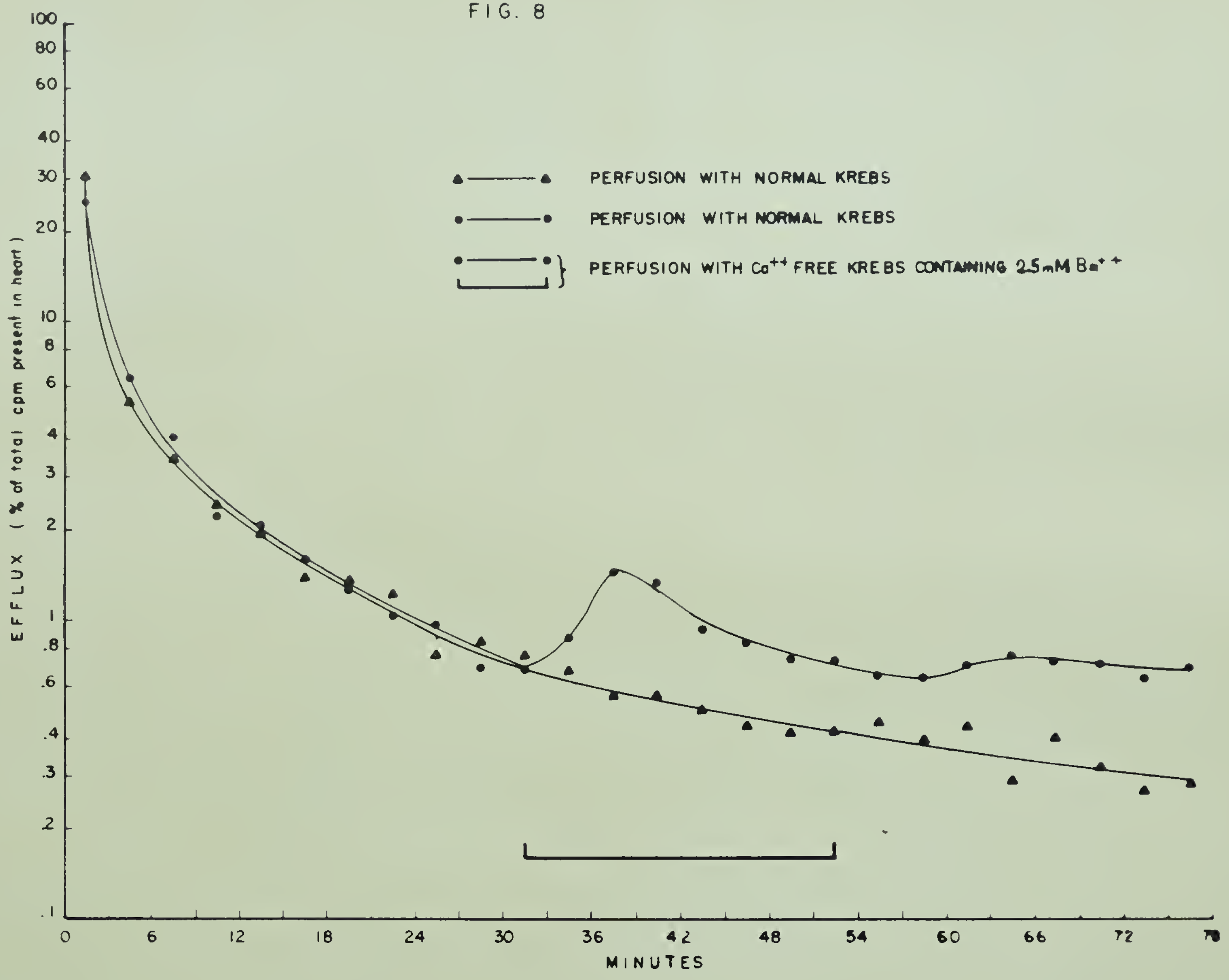

100
80
60
40
30
20
10
8
6
4
3
2
1
.8
.6
.4
.3
.2
.1
EFFLUX (% of total cpm present in heart)
0
6
12
18
24
30
36
42
48
54
60
66
72
78
MINUTES
PERFUSION WITH NORMAL KREBS
PERFUSION WITH NORMAL KREBS
PERFUSION WITH Ca^{++} FREE KREBS CONTAINING 2.5 mM Ba^{++}

FIGURE 8

Perfusion with 2.5 mM Barium substituted Krebs

After an initial period of perfusion with normal Krebs-bicarbonate solution test hearts were perfused with a calcium free Krebs medium containing 2.5 mM barium and then returned to normal Krebs. Control hearts received normal Krebs-bicarbonate solution throughout the experiment.

The corresponding data are given in Appendix II, Table 7.

FIG 9

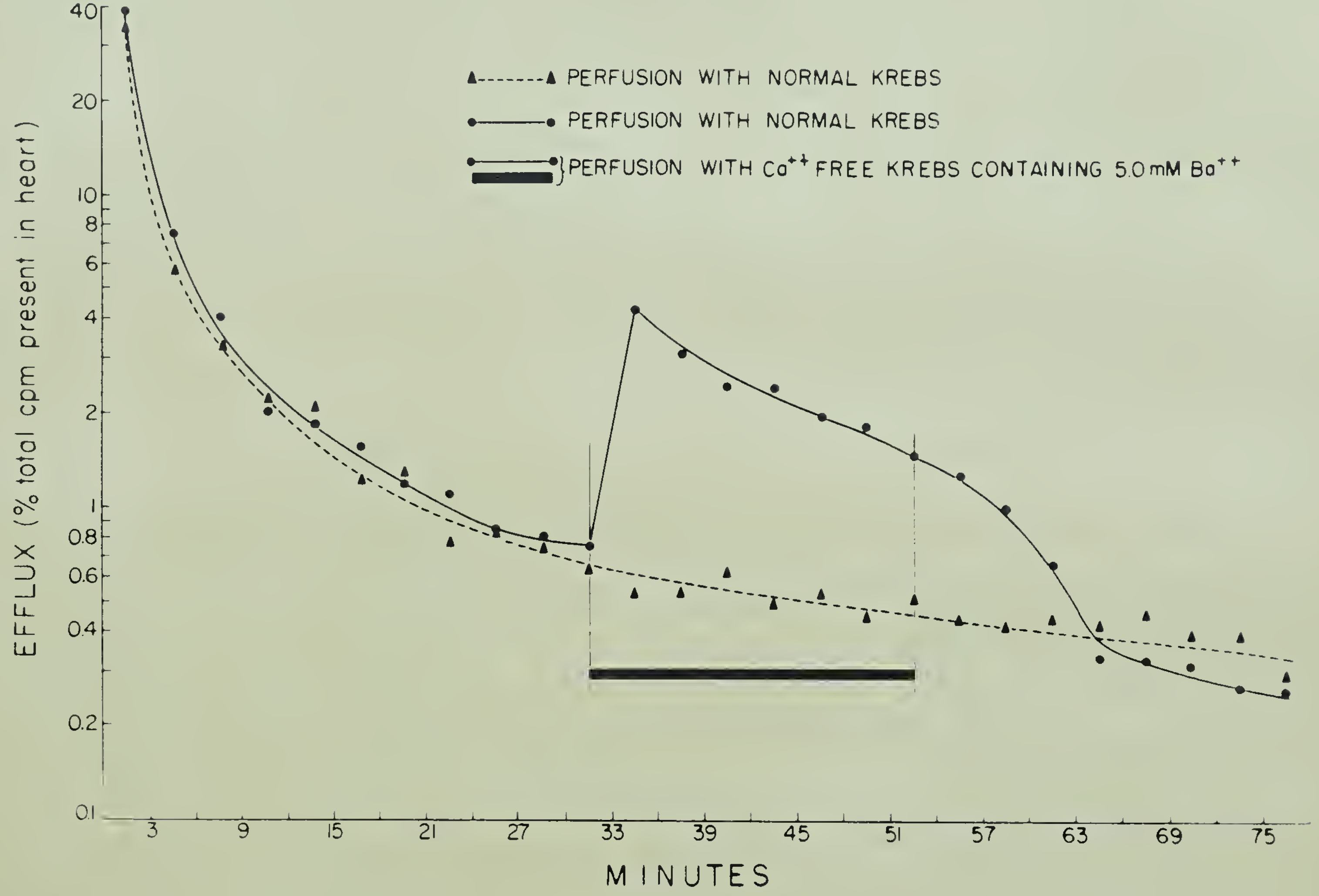

PERFUSION WITH NORMAL KREBS
PERFUSION WITH NORMAL KREBS
PERFUSION WITH Ca⁺⁺ FREE KREBS CONTAINING 5.0 mM Ba⁺⁺
EFFLUX (% total cpm present in heart)
40
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
0.1
3
9
15
21
27
33
39
45
51
57
63
69
75
MINUTES

FIGURE 9

Perfusion with 5.0 mM Barium substituted Krebs

After an initial period of perfusion with normal Krebs-bicarbonate solution test hearts were perfused with a calcium free Krebs medium containing 5.0 mM barium and then returned to normal Krebs. Control hearts received normal Krebs-bicarbonate solution throughout the experiment.

The corresponding data are given in Appendix II, Table 8.

FIG. 10

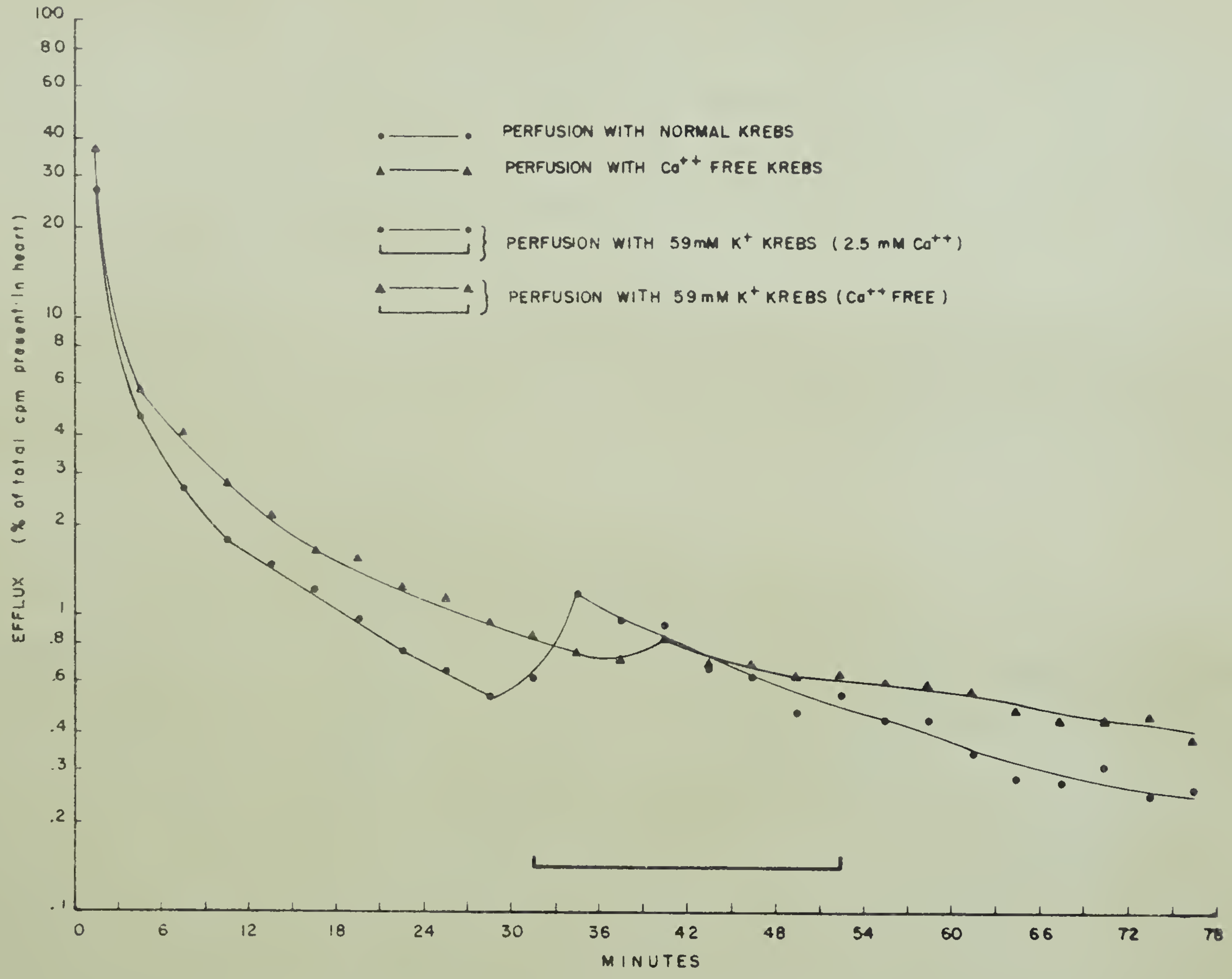

PERFUSION WITH NORMAL KREBS
PERFUSION WITH Ca^{++} FREE KREBS
PERFUSION WITH 59mM K^+ KREBS (2.5 mM Ca^{++})
PERFUSION WITH 59mM K^+ KREBS (Ca^{++} FREE)
EFFLUX (% of total cpm present in heart)
100
80
60
40
30
20
10
8
6
4
3
2
1
.8
.6
.4
.3
.2
.1
0
6
12
18
24
30
36
42
48
54
60
66
72
78
MINUTES

FIGURE 10

Potassium stimulation of release of ^{3}H-NA during perfusion with normal and calcium free Krebs

Excess potassium (59 mM), when introduced into hearts perfused with normal Krebs bicarbonate solution evoked release of ^{3}H-NA. Hearts perfused with a calcium free medium did not respond to excess potassium with an increase in efflux of ^{3}H-NA.

The corresponding data are given in Appendix II, Table 9.

FIG 11

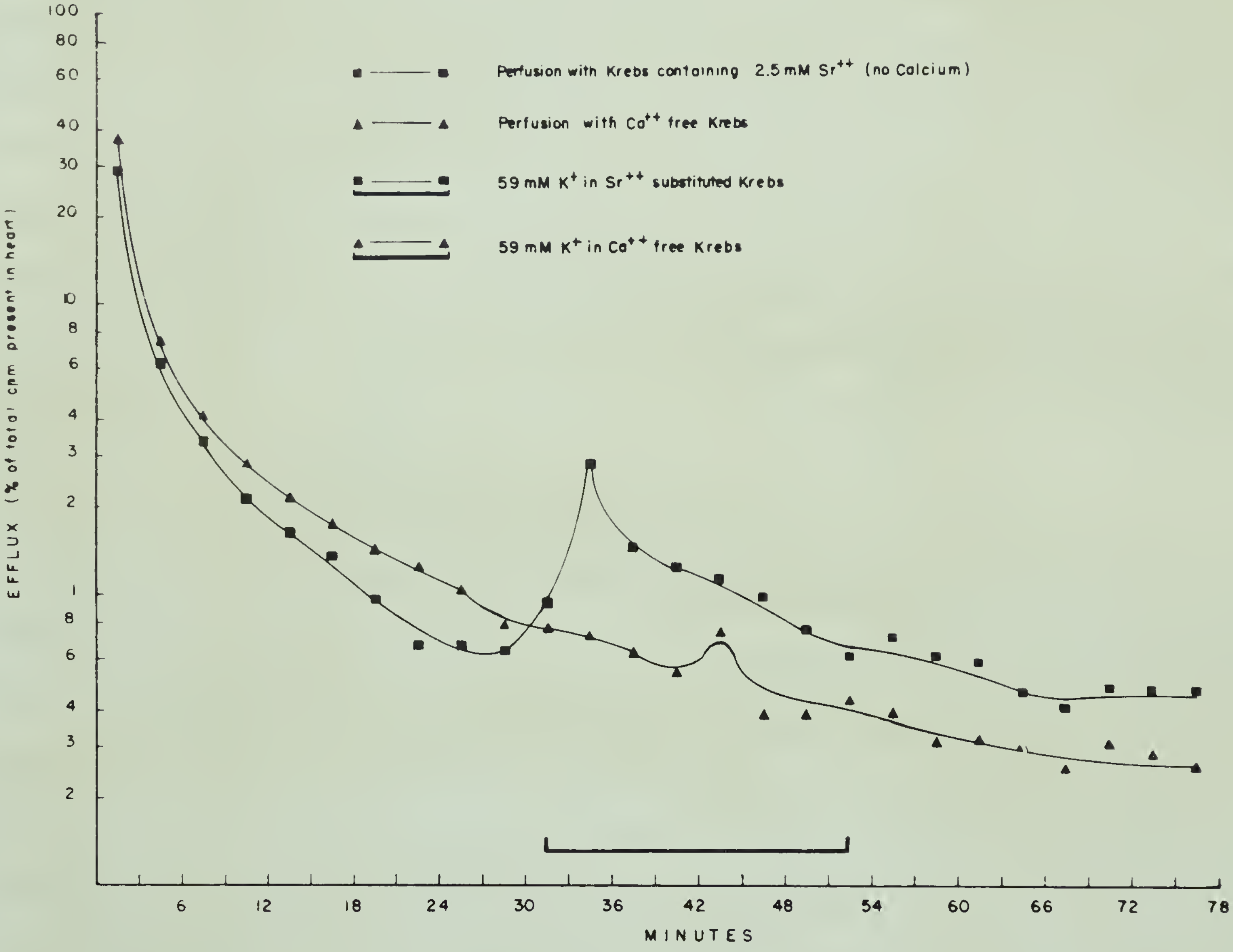

Perfusion with Krebs containing 2.5 mM Sr^{++} (no Calcium)
Perfusion with Ca^{++} free Krebs
59 mM K^+ in Sr^{++} substituted Krebs
59 mM K^+ in Ca^{++} free Krebs
EFFLUX (% of total cpm present in heart)
100
80
60
40
30
20
10
8
6
4
3
2
1
8
6
4
3
2
6
12
18
24
30
36
42
48
54
60
66
72
78
MINUTES

FIGURE 11

Strontium substituted Krebs and Stimulation by Potassium

After an initial period of perfusion with a calcium free medium containing 2.5 mM strontium addition of 59 mM potassium caused a substantial release of ^{3}H-NA. Addition of 59 mM potassium to hearts perfused with a calcium free medium did not stimulate a large increase in efflux of ^{3}H-NA.

The corresponding data are given in Appendix II, Table 10.

FIG. 12

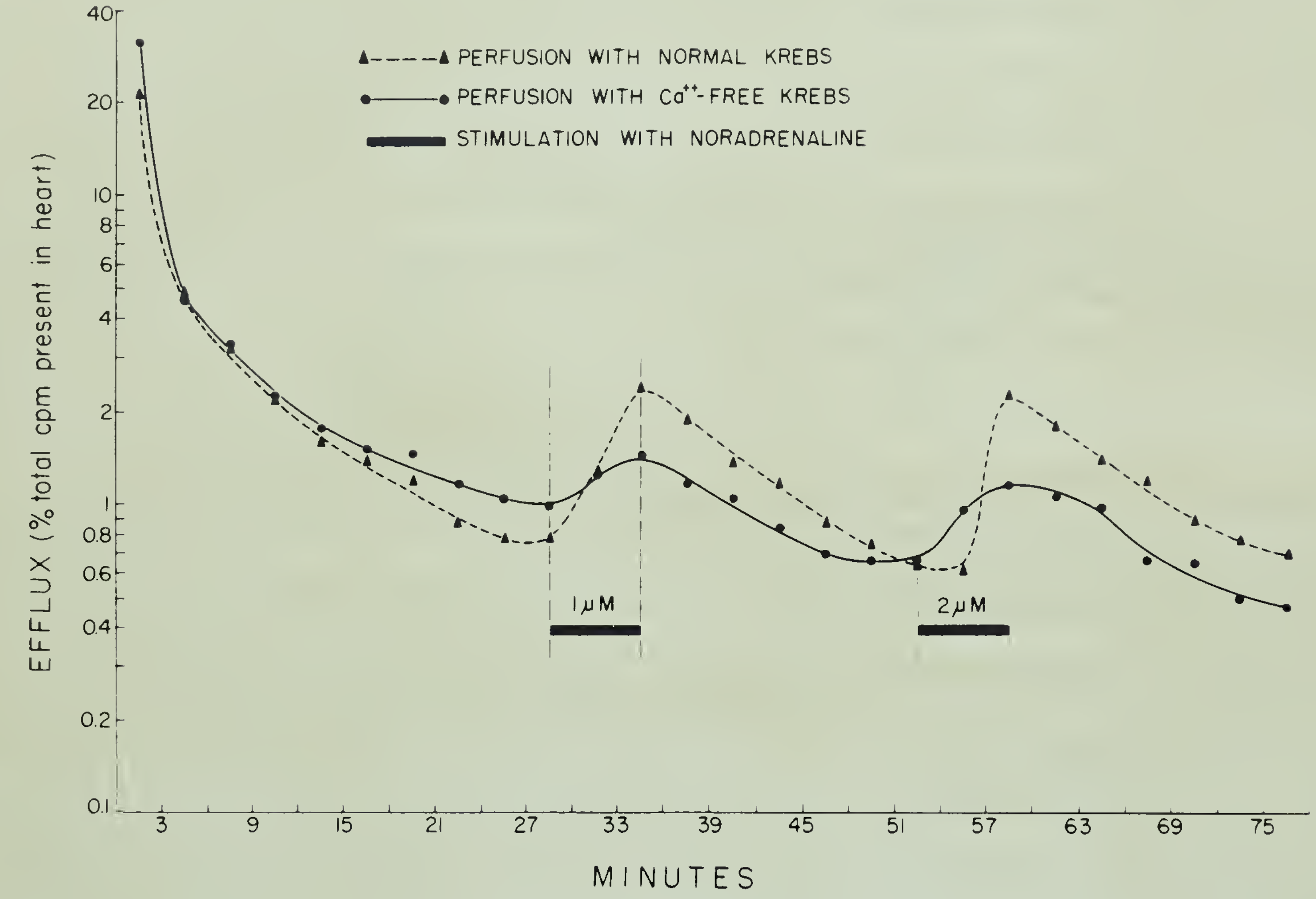

▲- - - -▲ PERFUSION WITH NORMAL KREBS
●——● PERFUSION WITH Ca^{++}-FREE KREBS
▬ STIMULATION WITH NORADRENALINE
EFFLUX (% total cpm present in heart)
40
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
0.1
1 μM
2 μM
3
9
15
21
27
33
39
45
51
57
63
69
75
MINUTES

FIGURE 12

Perfusion with calcium free Krebs and the stimulating action of Noradrenaline

l-NA infused at concentrations of 1 μM and 2 μM stimulated an increase in release of ^{3}H-NA during perfusion with normal Krebs-bicarbonate medium. If calcium was omitted from the perfusion medium the response to identical doses of l-NA was considerably less.

The corresponding date are given in Appendix II, Table 11.

FIG 13

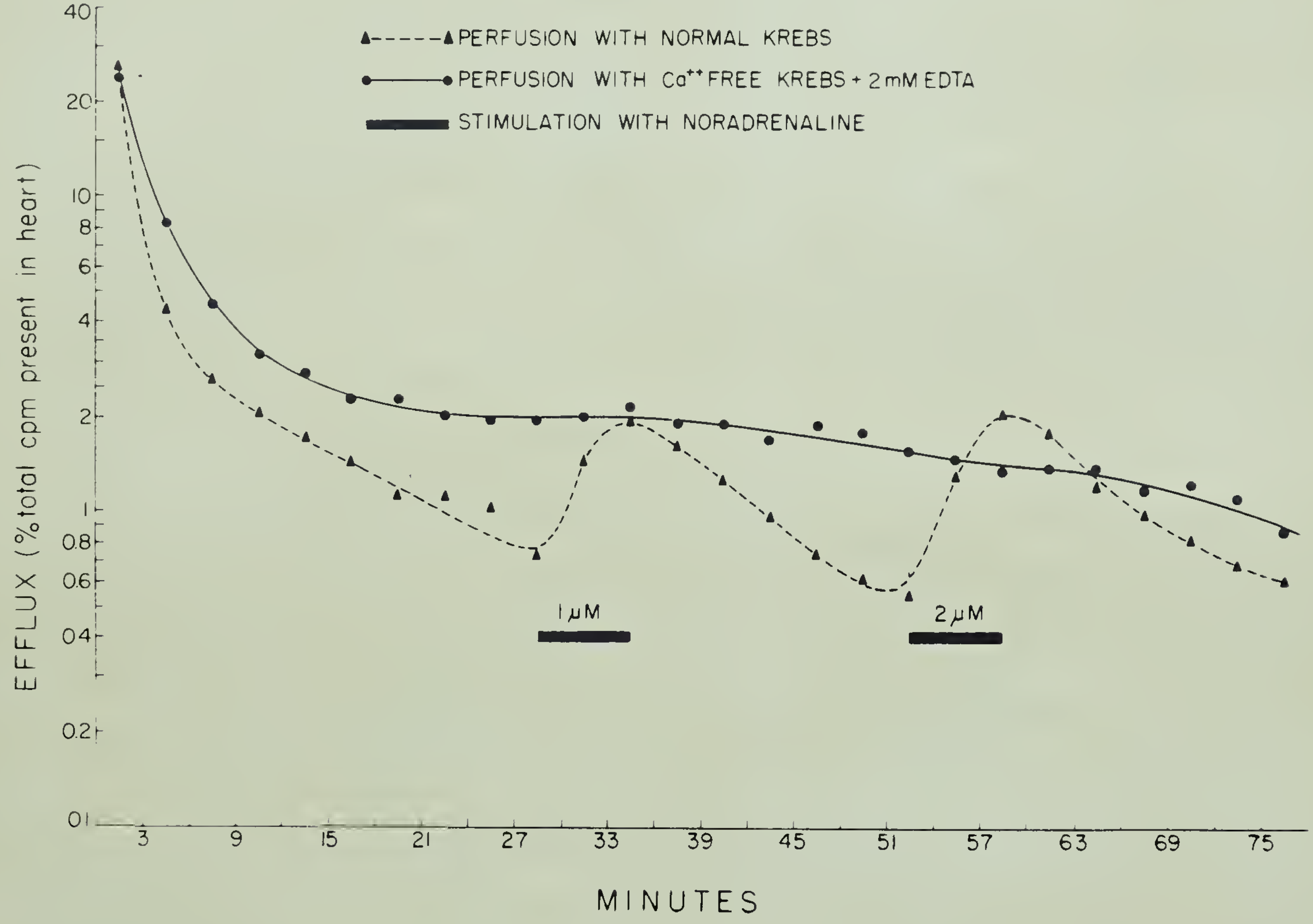

PERFUSION WITH NORMAL KREBS
PERFUSION WITH Ca++ FREE KREBS + 2 mM EDTA
STIMULATION WITH NORADRENALINE
EFFLUX (% total cpm present in heart)
40
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
01
1 μM
2 μM
3
9
15
21
27
33
39
45
51
57
63
69
75
MINUTES

FIGURE 13

Perfusion with calcium free Krebs + 2 mM EDTA and the stimulating action of Noradrenaline

During perfusion with normal Krebs-bicarbonate solution, l-NA at concentrations of 1 μM and 2 μM stimulated an increase in efflux of ^{3}H-NA. The same concentration of l-NA infused into hearts perfused by calcium free medium containing 2 mM EDTA produced no increase in efflux.

The corresponding data are given in Appendix II, Table 12.

FIG. 14

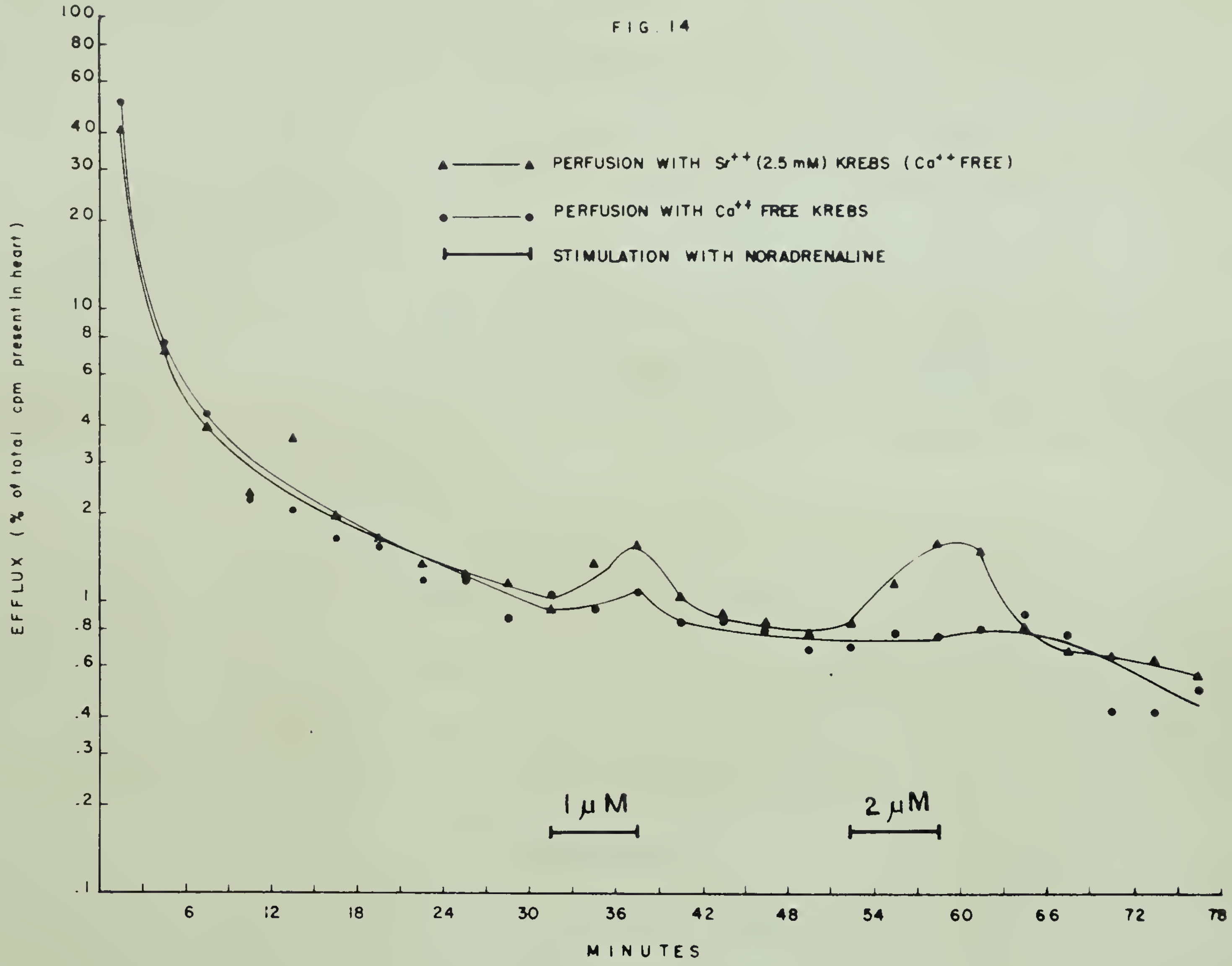

PERFUSION WITH Sr^{++} (2.5 mM) KREBS (Ca^{++} FREE)
PERFUSION WITH Ca^{++} FREE KREBS
STIMULATION WITH NORADRENALINE
1 µM
2 µM
EFFLUX (% of total cpm present in heart)
100
80
60
40
30
20
10
8
6
4
3
2
1
.8
.6
.4
.3
.2
.1
6
12
18
24
30
36
42
48
54
60
66
72
78
MINUTES

FIGURE 14

Strontium substituted Krebs and Noradrenaline Stimulation

The stimulating action of l μM and 2 μM l-NA is shown during perfusion with either strontium substituted calcium free Krebs or calcium free Krebs. The releasing action of l-NA was evident when the hearts were perfused with a strontium substituted medium, but a calcium free perfusing medium was not able to support an equivalent release of ^{3}H-NA.

The corresponding data are given in Appendix II, Table 13.

FIG. 15

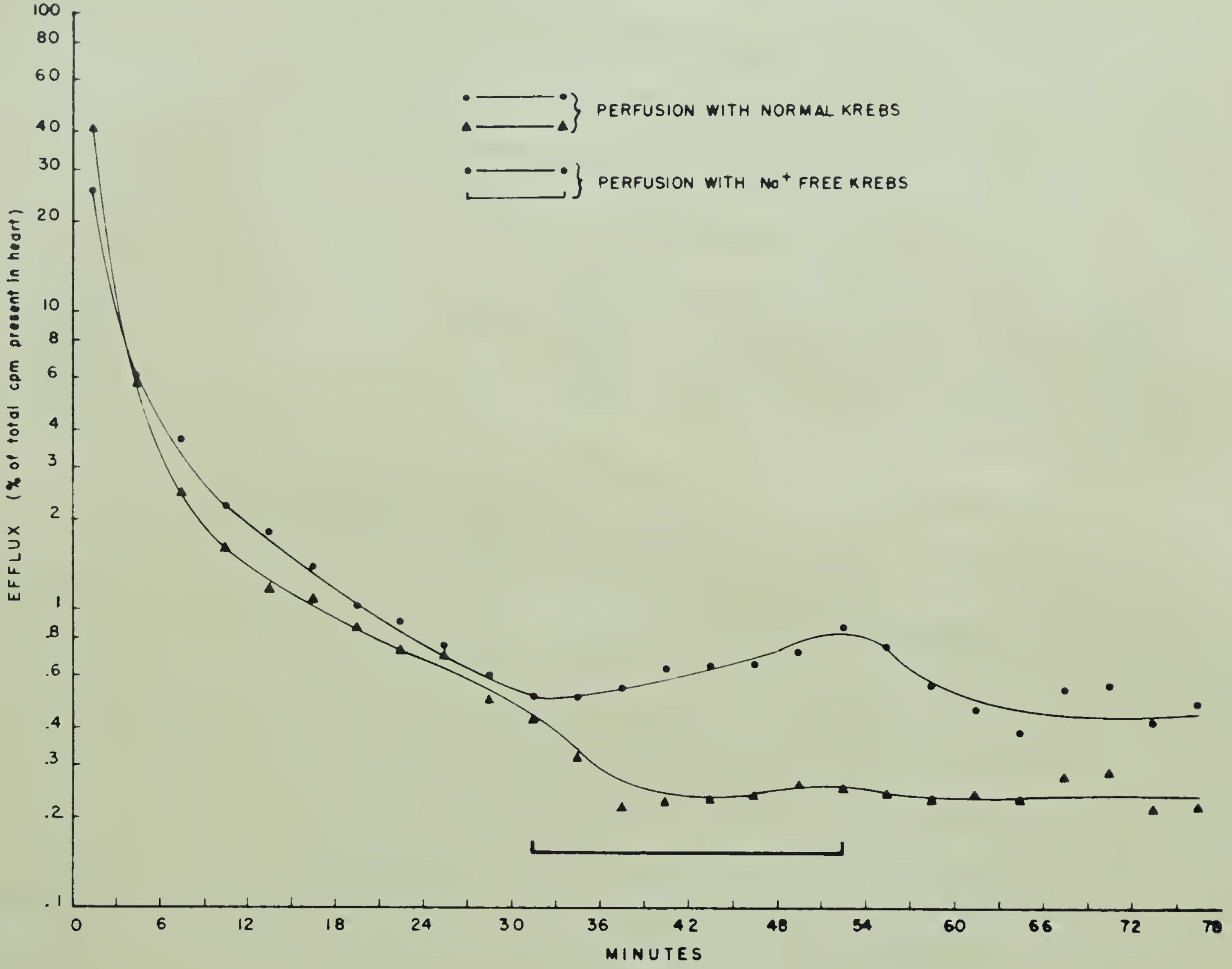

PERFUSION WITH NORMAL KREBS
PERFUSION WITH Na$^+$ FREE KREBS
EFFLUX (% of total cpm present in heart)
100
80
60
40
30
20
10
8
6
4
3
2
1
.8
.6
.4
.3
.2
.1
0
6
12
18
24
30
36
42
48
54
60
66
72
78
MINUTES

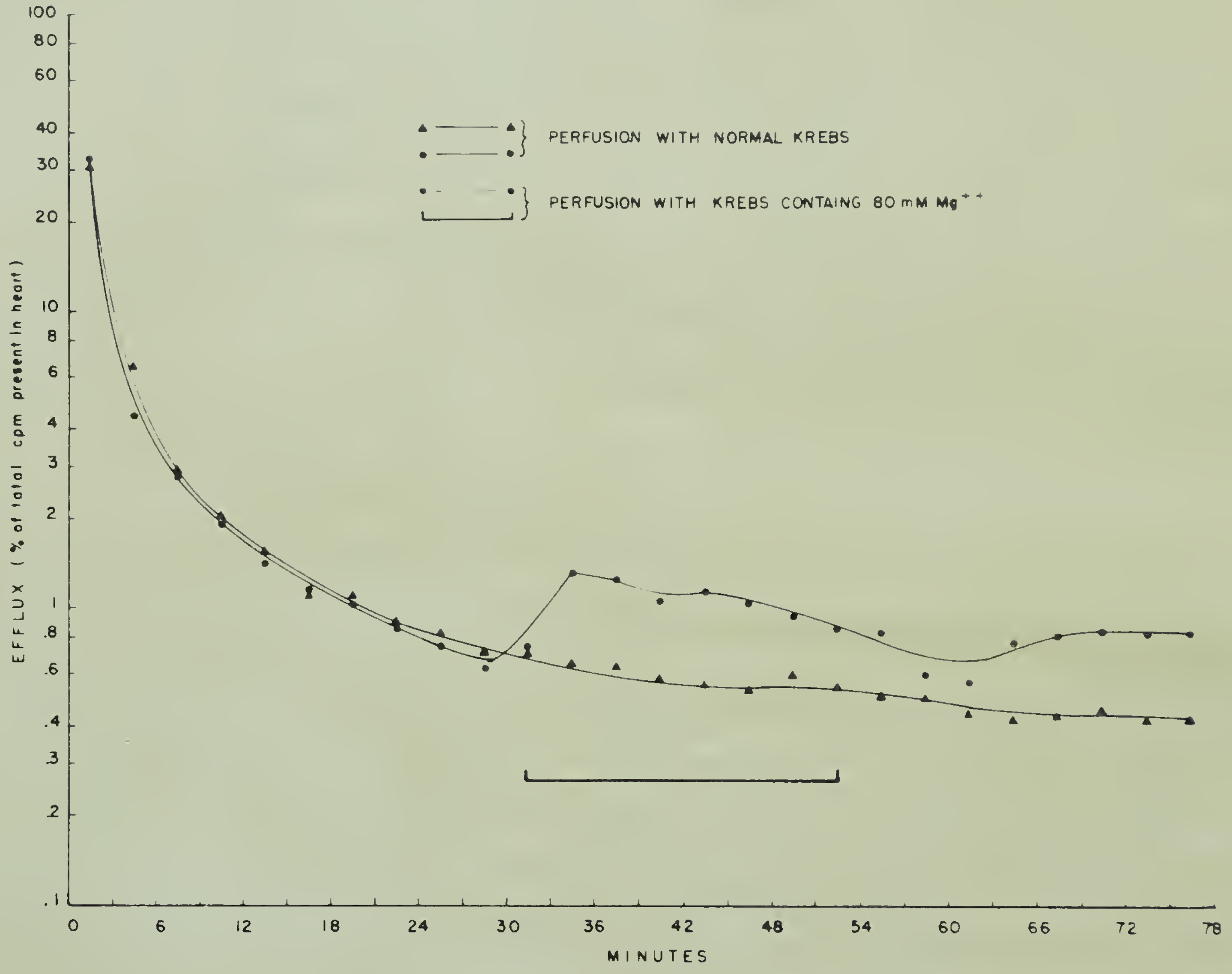
FIGURE 15

Perfusion with Low Sodium Krebs

Following perfusion with normal Krebs-bicarbonate solution a test period of low sodium Krebs (118 mM NaCl replaced by sucrose) caused a gradual increase in the release of ^{3}H-NA. Withdrawal of the low sodium Krebs medium caused the elevated efflux to approach control values obtained by perfusion with normal Krebs-bicarbonate solution.

The corresponding data are given in Appendix II, Table 14.

FIG. 16



PERFUSION WITH NORMAL KREBS
PERFUSION WITH KREBS CONTAING 80 mM Mg⁺⁺
EFFLUX (% of total cpm present in heart)
100
80
60
40
30
20
10
8
6
4
3
2
1
.8
.6
.4
.3
.2
.1
0
6
12
18
24
30
36
42
48
54
60
66
72
78
MINUTES

FIGURE 16

Perfusion with Excess Magnesium

In order to obtain a high concentration of magnesium (80 mM) practically all the sodium chloride had to be replaced by magnesium chloride. The experiment was therefore similar to Fig. 15 demonstrating the effect of low sodium on efflux. The elevation of efflux which occurred on switching to a medium containing a high magnesium concentration may in part be due to the lack of sodium from the perfusing medium.

The corresponding data are given in Appendix II, Table 15.

FIG. 17

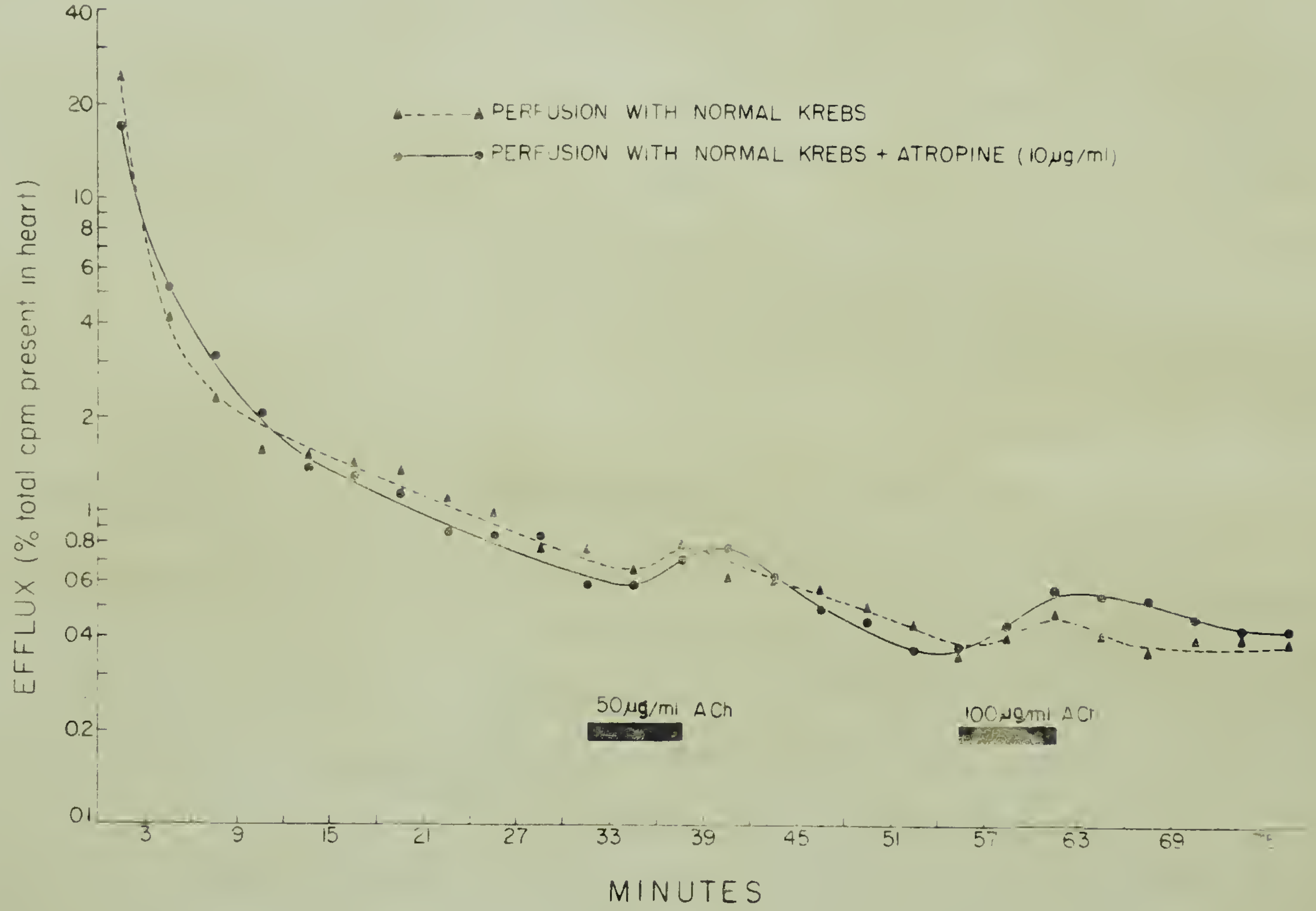

PERFUSION WITH NORMAL KREBS
PERFUSION WITH NORMAL KREBS + ATROPINE (10µg/ml)
EFFLUX (% total cpm present in heart)
40
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
0.1
3
9
15
21
27
33
39
45
51
57
63
69
50µg/ml ACh
100µg/ml ACh
MINUTES

FIGURE 17

The Releasing Action of Acetylcholine

Acetylcholine at concentrations of 50 μg/ml and 100 μg/ml introduced into atropinised and non-atropinised hearts caused a slight increase in efflux. In the non-atropinised hearts acetylcholine temporarily terminated the contractile activity of the heart whereas in the atropinised preparations an increase in chronotropic effect was observed.

The corresponding data are given in Appendix II, Table 16.

IV DISCUSSION

IV (a) Characteristics of NA Release

Tritiated NA has been utilised to label the endogenous stores of catecholamines; several methods have been employed to study its subsequent release. After infusion of ^{3}H-NA into cat's spleen Hertting & Axelrod (1961) were able to detect tritiated amine in the venous effluent during perfusion of the organ with blood from a donor cat that was free from radioactive material. Splenic nerve stimulation released ^{3}H-NA which was partially metabolised by Catechol-o-Methyl Transferase (COMT) partially returned to nerve endings and the remainder appeared in the venous effluent. Rosell et al (1963) have shown that stimulation of sympathetic nerves to an isolated skeletal muscle vascular bed exhibited a similar release phenomena. Spontaneous release from previously labelled organs has been studied in a number of ways. The decline in tissue radioactivity subsequent to labelling has been followed in rat heart by Brodie and Beaven (1963), in guinea pig heart by Crout (1964) and in rat brain by Glowinski and Axelrod (1965). In a more extensive study involving kinetic considerations of the decline in radioactivity (^{3}H-NA) using the above method in rat heart Beaven (1965) showed that there were probably at least 2 compartments contributing to the observed release of tritiated amine, one compartment (designated a mobile pool) appeared to be available for immediate release. Beaven postulated that a second compartment consisted of amine probably

bound in a catecholamine ATP complex (Potter et al 1962, Weiner and Jardetzky 1964) exchanged slowly with the mobile pool.

Efflux has also been studied using isolated perfused organs. Kopin et al (1962) demonstrated that ^{3}H-NA was concentrated in isolated perfused rat hearts. Hertting (1965) labelled the amine stores of isolated perfused rat hearts and analysed the subsequent efflux of tritiated amine. Immediately after cessation of infusion of ^{3}H-NA, radioactivity in the effluent was at a high level and declined in an exponential manner. However, in contrast to results obtained by following the decline in tissue content of ^{3}H-NA an initial rapid component to the efflux curve was noted. This phase reflected the loss of ^{3}H-NA from the extracellular fluid as shown by the use of ^{14}C-sorbitol and tritiated water (Iversen 1965b and Hertting 1965). As in the previous studies multiple compartmentalization was apparent from the shape of the efflux curve. Efflux curves of a similar nature were demonstrated in the present studies (Fig. 2) and by Titus et al (1966).

IV (b) Perfusion with Calcium Free Media

Removal of calcium from perfusion fluids is known to abolish mechanical activity (Ringer 1883) and only slightly modify electrical activity in heart muscle preparations (Mines 1913). The involvement of calcium in the contractile events in skeletal muscle is more complex. Edman and Grieve (1963) demonstrated that frog sartorius muscle after equilibration

with a calcium free medium did not respond either electrically or mechanically to electrical stimulation. Frank (1961) reported that muscles soeaked in calcium free media did not respond to potassium depolarisation. Consequently calcium is involved not only in production of the action potential but also in the process of excitation-contraction coupling that follow a depolarisation. In smooth muscle the rather complex picture may be summarised by saying that calcium is essential for the events which initiate and control the contractions of smooth muscle (see Bohr 1964 for review). According to Shanes (1958) low calcium media will unstabilise neuronal membranes and render them leaky. Calcium free media are also reported in certain instances to produce a fall in resting membrane potential (Edman and Grieve 1961, Weidman 1955).

Perfusion of rat hearts with calcium free media in the present series of experiments produced a slightly higher rate of efflux of ^{3}H-NA. The addition of 0.2 mM Na_2EDTA to a calcium free medium did not increase the release above that produced by calcium free Krebs alone. Fig. 3 shows the efflux of tritiated amine when 0.2 mM Na_2EDTA was added to calcium free Krebs in comparison to an efflux produced by normal Krebs perfusion containing 2.5 mM calcium. Figs. 10 and 12 also demonstrate the increased efflux caused by perfusion with calcium free medium as opposed to normal Krebs. However, addition of 2 mM Na_2EDTA to a calcium free perfusion medium markedly increased the rate of efflux of the tritiated amine. Na_2EDTA has equivalent ability to complex calcium and magnesium ions, therefore it was necessary to investigate whether the elevated release in the presence of Na_2EDTA could be mediated through an action involving magnesium, calcium or some other effect due to Na_2EDTA *per se*. The chelating agent EGTA has equivalent

ability to complex calcium and less ability to complex magnesium. Addition of either 2 mM Na_2EDTA or 2 mM EGTA to hearts perfused with a calcium free medium evoked release of approximately the same magnitude and suggest that the actions of Na_2EDTA were more likely on calcium and not magnesium in view of equal affinities of Na_2EDTA and EGTA for calcium ions. This, however, does not rule out the possibility that both Na_2EDTA and EGTA may be stimulating release through another independent unknown mechanism. In order to investigate further the releasing action of Na_2EDTA hearts were perfused with a calcium free Krebs medium containing 2 mM Na_2EDTA after initial perfusion with normal Krebs. Changing the perfusion medium to calcium free Krebs containing no Na_2EDTA the efflux of ^{3}H-NA decreased but in hearts perfused with calcium free Krebs containing 2 mM Na_2EDTA the release was sustained (Fig.4). Thus it appears that Na_2EDTA has some additional releasing action of its own when added to a calcium free perfusion medium. If calcium ions have the ability to stabilise a leaky membrane as was suggested by Shanes (1958) then replacement of calcium after a period of perfusion with calcium free medium should reduce the output of tritiated amine. Figs. 5 and 6 demonstrate this type of effect. Fig. 5 represents the effect illustrated from treatment of individual heart preparations and Fig. 6 the effect from continuous treatment of single hearts in the form of a crossover experiment. The elevation of spontaneous efflux of ^{3}H-NA observed in a calcium free medium in the present experiments has also been reported by Boullin and Brodie (1965) in the isolated vascular bed of the cat colon. Reduction of the calcium concentration in the perfusion fluid either with or without the addition of Na_2EDTA produced at least two effects in the present series of experiments. The increased efflux of ^{3}H-NA in calcium free media may be due to unstabilisation of the neuronal membrane.

Furthermore, the lack of stimulation observed in calcium free media may indicate a role of calcium in the intimate events linking stimulation with secretion of ^{3}H-NA, or may reflect the inability of the unstable neuronal membrane to adequately deal with the applied stimulus.

The actions of calcium at the granule level are by no means clear. In bovine chromaffin granules Phillipu and Schümann (1962) obtained a dose related release of catecholamines from 50-150% above control with increasing concentrations of calcium from 2.5 - 12.5 mM. Other workers (Hillarp 1958, Eade 1957, Carlsson *et al* 1963) observed a negligible release; however, they used lower concentrations in the range from 1.0 - 5.0 mM. Greenberg and Kolen (1966) studied catecholamine release from isolated rat chromaffin granules suspended in various electrolyte media in place of sucrose, as used by the above workers. They found that an increase in catecholamine release from granules occurred in NaCl-tris media and an inhibition in KCl-tris media with increasing concentrations of calcium from 0.0025 - 25 mM. However, the increase or decrease in release in either case was no more than 10-15% of control spontaneous release at 37° C which is in contrast to a more than 100 fold increase observed in the efflux of catecholamines from the intact adrenal gland observed by Douglas and Rubin (1964) in response to the addition of calcium to a calcium free perfusion medium. Schümann *et al* (1964) have reported that nerve granules from splenic nerve preparations were quite refractory to changes in calcium concentrations *in vitro*.

In view of the conflicting reports concerning the involvement of calcium at the granule level it is not possible to explain the effects observed in the present studies by reference to isolated granule activity alone. Furthermore, it is most likely that more than one role is fulfilled by calcium in the events which lead to the release of transmitter substances and endocrine secretions.

Iv (c) Substitution of Strontium and Barium for Calcium

A large number of multivalent cations including strontium have the ability to restore potassium induced contractures in skeletal muscle when added to calcium free solutions (Frank 1961). The introduction of barium chloride by means of a micropipette into a living muscle was shown by Heilbrunn and Wieninska (1947) to cause shortening of the myoplasm – an effect first observed with calcium chloride. Sodium, potassium and magnesium introduced in a similar manner were unable to elicit such a response. In certain smooth muscle preparations strontium and barium have the ability to maintain drug induced contractures in a calcium free medium (Daniel 1963). In a variety of species and preparations the contractibility of heart muscle can be maintained by substitution of strontium for calcium. A positive inotropic effect of strontium has been demonstrated on other cardiac muscle preparations stimulated at a given frequency (Garb 1951, Nayler and Emory 1962). The possibility that strontium could substitute for calcium in the release of ^{3}H-NA was investigated in the present experiments in two

different ways. First the stabilising action of adding calcium to a calcium free perfusion medium was extended by substituting strontium for calcium after the period of perfusion with calcium free medium. Secondly the ability of strontium to substitute for calcium during stimulated release of ^{3}H-NA was investigated (section IV (d)). In Fig. 7 the effect of adding strontium after a period of perfusion with calcium free Krebs + 2 mM Na_2EDTA is illustrated. In view of the additional stimulating effect of 2 mM Na_2EDTA alone (even in a calcium free medium) it is not possible to determine from these results alone whether or not strontium is able to substitute for calcium ions which may be involved in the spontaneous release of ^{3}H-NA. The matter is further discussed in Section IV (d).

The effects of barium on uterine smooth muscle are quite distinct from those of strontium. At concentrations similar to that of calcium in Krebs medium barium behaves as if it produces depolarisation and contracture (Daniel 1963). Takeshige et al (1964) demonstrated a stimulation of cat superior cervical ganglion when perfused with barium chloride media. The resultant depolarisation and postganglionic firing of the ganglion were attributed to any one of three possible mechanisms. The first considered the possibility that barium acted by releasing acetylcholine from preganglionic nerve endings (Douglas et al 1961, Teitel 1961). Secondly it was suggested the response to barium resulted from a penetration by the divalent cation of ganglionic cellular membranes. Thirdly the neuronal hyperirritability

caused by barium could be attributed to a decrease in the permeability of the neural membranes to potassium ions resulting in depolarisation and postganglionic firing (Shanes 1958). The stimulation of release of ^{3}H-NA observed in the present experiments (Figs. 8 and 9) may be due to any of the three explanations listed above. Douglas (1966) observed a release of catecholamines from the adrenal medulla in response to varying concentrations of barium in a calcium free medium. This secretory action of barium was postulated to be due to a displacement of membrane calcium and subsequent entry of barium ion into the chromaffin cell which then activated a catecholamine extrusion mechanism. Furthermore, Douglas (1966) also found that strontium, unlike barium, acted in a similar manner to calcium in that strontium required the presence of a stimulatory agent (e.g. excess potassium) in order to promote extrusion of catecholamines.

IV (d) Calcium involvement in stimulated release

High concentrations of potassium ion (59 mM), which may be assumed to depolarise, caused a stimulated release of ^{3}H-NA above the control value obtained by perfusion with normal Krebs (Fig. 10). Furthermore, removal of calcium from the perfusion medium caused an elevation of the spontaneous efflux of tritiated amine but the response to potassium stimulation in the calcium free medium was almost completely abolished. Although not illustrated, further experiments demonstrated that the potassium induced response was abolished only when at least thirty minutes of calcium free Krebs

perfusion preceeded the stimulus. The response to 59 mM potassium was still evident when a calcium containing medium (2.5 mM) was perfused prior to the stimulus despite the fact that the stimulus was introduced in a calcium free medium (i.e. 59 mM potassium, calcium free Krebs). It is unlikely that the nerve endings were depleted of ^{3}H-NA after perfusion with calcium free medium in view of the results obtained in the extended crossover experiment (Fig. 6).

In view of the similarities noted above (Sections IV (a), (b) and (c) in the actions of strontium and calcium it is interesting to observe the similarity in efflux curves obtained by perfusion with normal Krebs (containing 2.5 mM calcium) and strontium substituted Krebs (containing no calcium but 2.5 mM strontium). Figs. 10 and 11 also show that calcium free Krebs perfusion resulted in an increased spontaneous release above the control but did not permit an increased efflux of tritiated amine during stimulation. Further evidence for the critical involvement of calcium in the release of ^{3}H-NA was obtained from the use of another stimulating agent. Laevo-noradrenaline has been shown to cause a stimulated increase in efflux of ^{3}H-NA from isolated heart preparations (Daly et al 1966). When introduced into normal Krebs perfusion medium l-noradrenaline caused an increase in efflux as shown in Fig. 12. Hearts perfused with a calcium free medium released less of the tritiated amine in response to the stimulating dose of NA. If 2 mM Na_2EDTA was added to the calcium free perfusion

medium the response to NA was abolished (Fig. 13). As in the experiments involving potassium stimulation, the releasing effect of NA was also evident in a strontium substituted perfusion medium. Hukovic and Muscholl (1962) using isolated perfused rabbit hearts have reported that lowering the calcium concentration of the perfusion medium to one quarter of its conventional value approximately halved the output of NA in response to stimulation of cardiac nerves.

The results above involving characteristic ionic dependence of stimulated release are also in agreement with the work of Boullin and Brodie (1965) who demonstrated an increased spontaneous efflux of ^{3}H-NA from the isolated cat colon when perfused with a calcium free medium. They also showed that stimulation of the inferior mesenteric nerve did not release tritiated amine when a calcium free medium perfused the tissue. A further similarity was the stimulatory action of barium ion on the release of labelled NA from the isolated cat colon. Kirpekar et al (1966) have observed an increased efflux of NA in response to stimulation of the splenic nerve in an isolated spleen preparation. When a calcium free medium perfused the tissue nerve stimulation reduced the NA efflux by 90%.

The involvement of calcium ions in the release of catecholamines from the adrenal medulla has been reviewed recently by Douglas (1966). He found that acetylcholine, the physiological stimulus causing the chromaffin cell to release catecholamines, was ineffective in the absence of calcium;

that the response to acetylcholine increased with increasing calcium concentrations over a wide range; that acetylcholine increased the Ca^{45} uptake in the medulla and that calcium, strontium and barium were capable of evoking catecholamine secretion in appropriate circumstances in the absence of medullary secretogogues. In summary, it was concluded that the influx of calcium was responsible for initiating catecholamine release. The present studies do not substantiate all of the conclusions of Douglas (1966). When a calcium containing medium was introduced after perfusion of hearts with either calcium free or calcium free + 2 mM Na_2EDTA Krebs, no stimulation of release was observed. However, agreement is found with results which suggest the necessity for calcium at some site during stimulated release with either potassium or NA (Figs. 10, 12 and 13). This may represent calcium bound within the cell membrane or a granule complex, and involved in the extrusion of either the ruptured granule or the whole granule. It may also represent a functional component of the extrusion process at the nerve membrane or be involved in the mobilisation of the granule-bound and mobile pools of NA within the nerve terminal.

IV (e) Further ionic requirements during release

The ionic requirements for the release of acetylcholine from

motor nerve terminals has been recently shown by Gage and Quastel (1965) and Birks and Cohen (1965) to be dependent on external sodium concentration. The results of Birks and Cohen (1965) demonstrated that reduction of the extracellular sodium ion concentration in a low calcium medium increased the spontaneous release of transmitter from motor nerve terminals. When the extracellular sodium content of the perfusion medium was reduced in the present experiments an increased efflux of tritiated amine was demonstrated (Fig. 15). Birks and Cohen (1965) suggest that the stimulating action of a decrease in sodium in a low calcium media in motor nerve terminals may be related to changes in calcium availability within the nerve terminal. In a normal calcium containing media the stimulating effect of low sodium is less striking or absent. They assume that the amount of acetylcholine released is ultimately determined by the amount of calcium displaced from the motor nerve terminal. Lüttgau and Niedergerke (1958) proposed that the increase in contractile response of heart muscle that occurs in sodium deficient solutions was a function of the availability of membrane-bound calcium, the inward movement of which initiates contraction. In the absence of external sodium more sites were readily saturable by calcium and hence able to increase the contractile response of the muscle. In the motor nerve terminal it was postulated (Birks and Cohen 1965) that a similar situation existed and that in low sodium media, calcium was more readily available to initiate release because of its lack of competition with sodium for binding sites.

In a low sodium media containing normal amounts of calcium it is plausible that release may be enhanced through greater calcium binding and subsequent mobilisation in a similar manner to that postulated for release of acerylcholine.

High concentrations of magnesium ion have been shown by Douglas and Rubin (1964) to have little effect on spontaneous release of catecholamine from adrenal glands perfused with either calcium containing or calcium free Krebs. However, magnesium was shown by these workers to be inhibitory on the stimulated release of catecholamines produced by either potassium or acetylcholine. On the other hand Schümann and Philippu (1963) have observed stimulant effects of magnesium on catecholamine release. Fig. 16 shows an increased efflux of ^{3}H-NA when high concentrations of magnesium were employed. It appears that magnesium has the ability to enhance efflux but in order to attain a high concentration of magnesium and maintain tonicity it is necessary to replace practically all the sodium. Thus the experiment is similar to the one mentioned above (Fig. 15) using a sodium free solution. The explanation for this additional release in sodium free media containing excess magnesium is not apparent.

IV (f) Release of tritiated Noradrenaline and Acetylcholine

In atropinised isolated mammalian hearts acetylcholine is known to cause increases in rate and tension (Hoffman *et al* 1945; Richardson *et al*

1959). In these preparations a release of NA has been demonstrated (Angelakos and Bloomquist 1965). The release of ^{3}H-NA demonstrated in Fig. 17 is consistent with the above findings and illustrates one further point. Acetylcholine infusion into non-atropinised preparations caused a cessation of mechanical activity in contrast to the increase in rate produced in atropinised preparations. The release of ^{3}H-NA in response to acetylcholine in each case (i.e. atropinised or non-atropinised) was of the same order of magnitude and eliminates the possibility that observed release in instances where increased mechanical activity occurs may be due to physical compression of tissue leading to increased efflux. The site of action of acetylcholine is not evident from the present results. In the adrenal medulla Douglas (1966) suggested that acetylcholine induced secretions of catecholamines were due to an increase in permeability of the chromaffin cell membrane and subsequent entry of calcium ions. Normal Krebs medium employed in the present studies contains calcium and other cations and without further evidence it is not possible to speculate that calcium ion alone was the only one involved in acetylcholine induced release of tritiated amine.

V SUMMARY AND CONCLUSIONS

1. The rate of efflux of ^{3}H-NA from isolated perfused rat hearts was found to be dependent upon the ionic composition of the perfusion medium.

2. The rate of release of ^{3}H-NA was elevated by the removal of calcium from the perfusion medium. Addition of varying quantities of Na_2EDTA to a calcium free medium elevated the release above that caused by calcium free Krebs alone. The increase in efflux caused by perfusing the hearts with calcium free media was reversible upon replacing calcium. The stimulating action of Na_2EDTA was also reversible when the chelating agent was removed from the perfusion media.

3. Stimulated release of ^{3}H-NA either by the addition of l-noradrenaline or excess potassium ions was reduced in the former and abolished in the latter case when calcium was omitted from the perfusion medium. Strontium ions were able to substitute for calcium in maintaining stimulated release with either of the above agents. Substitution of barium for calcium in the perfusing medium caused a marked stimulation of efflux of ^{3}H-NA.

4. Perfusion with Krebs containing a low concentration of sodium (tonicity being maintained with sucrose) increased the efflux of ^{3}H-NA above control levels obtained by perfusion with normal Krebs. A more

pronounced release of ^{3}H-NA was demonstrated in response to perfusion with a low sodium medium containing a high concentration of magnesium.

5. Acetylcholine infused at concentrations of 50 μg/ml and 100 μg/ml caused an approximately equal increase in efflux of ^{3}H-NA in atropinised and non-atropinised preparations. It was concluded therefore that the rate of beating of the hearts was not a factor in determining the rate of release of the tritiated amine.

BIBLIOGRAPHY

ANGELAKOS, E.T. and BLOOMQUIST, E. (1965). Release of norepinephrine from isolated hearts by acetylcholine. Arch. Int. de Physiol. et de Biochim, 73, (3) 397-402.

ANGELAKOS, E.T., FUXE, K. and TORCHIANA, M.L. (1963). Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea pig hearts. Acta physiol. scand., 59, 184-192.

BACQ, Z.M. (1947). La substance sympathicomimétique des extraits de tissus. Compte. rend. Soc. de biol., 141, 963-964.

BANKS, P. and BLASCHKO, H. (1966). Chromaffin Tissue. In Second Symposium on Catecholamines. Held in Milan 1965, ed Acheson, G.H., Pharmacol. Revs., 18, 1, 453-455.

BEAVEN, M.A. (1965). Adv. in Tracer Methodology. Plenum Press, N.Y., 2, 243-252.

BIRKS, R.I. and COHEN, M.W. (1965). Effects of sodium on transmitter release from frog motor nerve terminals. In Muscle, ed Daniel, E.E., Paul, W.M., Kay, C.M. and Monckton, G. Pergamon Press, 403-420.

BLASCHKO, H. and WELCH, A.D. (1953). Localization of adrenaline in cytoplasmic particles of the bavine adrenal medulla. Arch. Exp. Path. Pharmak., 219, 17-22.

BOHR, D.F. (1964). Electrolytes and smooth muscle contraction. Pharmacol. Rev., 16, 85.

BOULLIN, D.J. (1965). Effect of divalent ions on release of ^{3}H-noradrenaline by sympathetic nerve stimulation. J. Physiol., 183, 76-77 P.

BOULLIN, D.J. and BRODIE, B.B. (1965). Effect of cations on the output of H^3-norepinephrine from sympathetic nerves. Pharmacologist, Fall 1965, 168.

BRODIE, B. and BEAVEN, M.A. (1963). Neurochemical Transducer System. Med. Exp. 8, 320-351.

BURN, J.H. and GIBBONS, W.R. (1964). The part played by calcium in determining the response to stimulation of sympathetic postganglionic fibres. Brit. J. Pharmacol., 22, 540-548.

CALLINGHAM, B.A. and CASS, R. (1963). A modification of the butanol extraction method for the fluormetric assay of catecholamines in biological material. J. Pharm., Pharmacol., 15, 699-700.

CARLSSON, A., HILLARP, N.A. and WALDECK, B. (1963). Analysis of the Mg^{++}- ATP dependent storage mechanism in the amine granules of the adrenal medulla. Acta physiol. scand., Suppl. 215, 59, 1.

CAMPOS, H.A. and SHIDEMAN, F.E. (1962). Subcellular distribution of catecholamines in the dog heart. Int. J. Neuropharmacol., 1, 13-22.

CANNON, W.B. and URIDIL, J.E. (1921). Studies on the conditions of activity in endocrine glands. VIII Some effects on the denervated heart of stimulating the nerves of the liver. Am. J. Physiol., 58, 353-354.

CANNON, W.B. and ROSENBLUETH, A. (1933). Sympathin E and Sympathin I. Am. J. Physiol., 104, 557-574.

COOPER, T. (1965). Physiologic and pharmacologic effects of cardiac denervation. Fed. Proc., 24, 6, 1428-1431.

CROUT, J.R. (1964). The uptake and release of ^{3}H-norepinephrine by the guinea pig heart in vivo. Arch. Exp. Path. Pharmakol., 248, 85-98.

DAHLSTRÖM, A., FUXE, K., MYA-TU, M. and ZETTERSTRÖM, B.E.M. (1965). Observations on adrenergic innervation of dog heart. Am. J. Physiol., 206, 4, 689-692.

DALY, J.W., CREVELING, C.P. and WITKOP, B. (1966). The chemo-release from mouse hearts. Structure activity relationships I - Sympathomimetic and related amines. J. Med. Chem., 9, 273-280.

DANIEL, E.E. (1963). On the roles of calcium, strontium and barium in contraction and excitability of rat uterine muscle. Arch. Int. de Pharmacodyn., 146, 298-349.

DENGLER, H.J., SPIEGEL, H.E. and TITUS, E.D. (1961). Effects of drugs on uptake of isotopic norepinephrine by cat tissues. Nature, (Lond.), 191, 816-817

DENGLER, H.J., MICHAELSON, I.A., SPIEGEL, H.E. and TITUS, E. (1962a). The uptake of labelled norepinephrine by isolated brain and other tissues of the cat. Int. J. Neuropharmacol., 1, 23-38.

DENGLER, H.J., WILSON, C.W.M., SPIEGEL, H.E. and TITUS, E. (1962b). Uptake of norepinephrine by isolated pineal bodies. Biochem. Pharmacol., 11, 795-801.

DENGLER, H.J. (1965). Effect of drugs on monoamine uptake in isolated tissues. Proc. 2nd Int. Pharmacol. Meeting, ed. Koelle, G.B., Douglas, W.W. and Carlson, A., Vol. 3, pp. 261-275, Pergamon Press, Oxford.

DE ROBERTIS, E. and PELLEGRINO de IRALDI, A. (1961). A Plurivesicular component in adrenergic nerve endings. Anat. Rec., 139, 299.

DE ROBERTIS, E. and PELLEGRINO de IRALDI, A. (1961a). Plurivesicular secretory process and nerve endings in the pineal gland of the rat. J. Biophys. Biochem, Cytol., 10, 361-372.

DOUGLAS, W.W., LYWOOD, D.W. and STRAUB, R.W. (1961). The stimulant effect of barium on the release of acetylcholine from the superior cervical ganglion. J. Physiol., 156, 515-522.

DOUGLAS, W.W. and RUBIN, R.P. (1961a). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J. Physiol., 159, 40-57.

DOUGLAS, W.W. and RUBIN, R.P. (1961b). The role of calcium in adrenal medullary secretion evoked by acetylcholine or potassium. J. Physiol., 159, 24-25 P.

DOUGLAS, W.W. and POISNER, A.M. (1961c). Stimulation of uptake of Ca^{45} in the adrenal gland by acetylcholine. Nature, (Lond.), 192, 1299.

DOUGLAS, W.W. and POISNER, A.M. (1962). On the mode of action of acetylcholine in evoking adrenal medullary secretion, increased uptake of calcium during the secretory response. J. Physiol., 162, 385-392.

DOUGLAS, W.W. and RUBIN, R.P. (1964). The stimulant action of barium on the adrenal medulla. Nature, (Lond.), 203, 305-307.

DOUGLAS, W.W. (1965). Acetylcholine as a secretogogue, Ca^{++} dependent links in "stimulus secretion coupling" at the adrenal medulla and submaxillary gland. Douglas, W.W. Pharmacol. of Cholin. and Adren. Trans., Pergamon, 2nd Int. Pharm. Meet. Vol. 3.

DOUGLAS, W.W. (1966). Calcium-dependent links in stimulus - secretion coupling in the adrenal medulla and neurohypophysis. In Mechanism of Release of Biogenic amines. Proc. of an Internation Wenner-Gren Symposium held in Stockholm February 1965. Pergamon Press, 267-290.

EADE, N.R. (1957). The release of catecholamines from isolated chromaffin granules. Brit. J. Pharmacol., 12, 61-65.

EDMAN, K.A.P. and GRIEVE, D.W. (1961). The role of calcium and zinc in the electrical and mechanical responses of frog sartorius muscle. Experientia., 17, 557-558.

ERÄNKÖ, O. (1955). Histochemistry of noradrenaline in the adrenal medulla of rats and mice. Endocrinology, 57, 363-368.

EULER, U.S. von. (1948). Identification of the sympathomimetic ergone in adrenergic nerves of cattle (sympathin N) with laevo-noradrenaline. Acta physiol. scand., 16, 63-74.

EULER, U.S. von, HAMBERG, U. and HEUNER, S. (1951). B-(e:4-Dihydroxyphenyl) ethylamine (hydroxytryptamine) in normal human urine. Biochem. J., 49, 655-663.

EULER, U.S. von and HILLARP, N - Å. (1956). Evidence for the presence of noradrenaline in submicroscopic structures of adrenergic axons. Nature, (Lond.), 177, 44-45.

EULER, U.S. von and LISHAJKO, F. (1961). Noradrenaline release from isolated nerve granules. Acta physiol. scand., 51, 193-203.

EULER, U.S. von and LISHAJKO, F. (1962). Release and uptake of catecholamines in nerve storage granules in relation to adrenergic neurotransmission. Arch. Int. Pharmacodyn., 139, 276-280.

EULER, U.S. von and LISHAJKO, F. (1965). Effect of drugs on the storage granules of adrenergic nerves. Proc. 2nd. Int. Pharmacol. Meeting ed. Koelle, G.B., Douglas, W.W. and Carlsson, A. Vol. 3, pp 245-260, Pergamon Press, Oxford 1965.

EULER, U.S. von (1966). Twenty years of noradrenaline. In Second Symposium on Catecholamines. Held in Milan 1965, ed. Acheson, G.H. Pharmacol. Revs., 18, 1, 29-38.

FALCK, B. (1962). Observations on the possibilities of the cellular localisation of monoamines by a fluorescence method. Acta physiol. scand., 56, Suppl. 197.

FALCK, B., HILLARP, N - Å., THIEME, G. and TORP, A. (1962). Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem., 10, 348-354.

FRANK, G.B. (1961). Role of extracellular calcium ions in excitation-contraction coupling in skeletal muscle. In Biophysics of Physiological and Pharmacological actions, ed. Shanes, A. pp 293-307, Publ. No. 69, Amer. Ass. Advanc. Sci., Washington D.C.

FUXE, K. (1965). The distribution of monamine nerve terminals in the central nervous system. Acta physiol. scand., 64, Suppl. 247.

GAGE, P.W. and QUASTEL, D.M.J. (1965). Influence of sodium ions on transmitter release. Nature, (Lond.), 206, 4988, 1047-1048.

GARB, S. (1951). The Effects of potassium, ammonium, calcium, strontium and magnesium on the electrogram and myogram of mammalian heart muscle. J. Pharmacol. Exp. Therap., 101, 317-326.

GILLESPIE, J.S. and KIRKEPAR, S.M. (1963). Removal of noradrenaline from the blood following through the spleen. J. Physiol., 169, 100P.

GLOWINSKI, J. and AXELROD, J. (1965). Effect of drugs on the uptake, release and metabolism of ^{3}H-norepinephrine in the rat brain. J. Pharmacol. Exp. Therap., 149, 1, 43-49.

GLYNN, I.M. (1957). The actions of cardiac glycosides on sodium and potassium movements in human red cells. J. Physiol., 136, 148-173.

GRILLO, M.A. and PALAY, S.L. (1962). Granule containing vesicles in the autonomic nervous system. In Electron Microscopy, Fifth International Congress for Electron Microscopy, ed. Breese, S.S., Jr., 2, pp U 1, Academic Press N.Y., 1962.

GREENBERG, R. and KOLEN, C.A. (1966). Effects of acetylcholine and calcium ions on the spontaneous release of epinephrine from catecholamine granules. Proc. Soc. Exptl., Biol. and Med., 121, No. 4, 1179.

HAGEN, P. (1959). The storage and release of catecholamines. In Ciba Symposium on Catecholamines, 361-374, Williams & Wilkins Co.

HEILBRUNN, L.V. and WIERCINSKI, F.J. (1947). The action of various cations on muscle protoplasm. J. cell. comp. Physiol., 29, 15-32.

HERTTING, G. and AXELROD, J. (1961). Fate of tritiated noradrenaline at the sympathetic nerve endings. Nature, (Lond.), 192, 172-173.

HERTTING, G. (1965). Effect of drugs and sympathetic denervation on noradrenaline uptake and binding in animal tissues. Pharmacol. of Cholin. and Adren. Trans., Pergamon, 2nd Int. Pharm. Meet., 277 - 289.

HERTTING, G., AXELROD, J. and WHITBY, L.G. (1965). Effect of drugs on the uptake and metabolism of ^{3}H-norepinephrine. J. Pharmacol. exp. Ther., 134, 145-153.

HILLARP, N - Å, LAGERSTEDT, S. and NILSON, B. (1953). The isolation of a granular fraction from the suprarenal medulla containing the sympathominetic catecholamines. Acta physiol. scand., 29, 251-263.

HILLARP, N - Å. (1955). Adenosine triphosphate in the adrenal medulla of the cow. Nature, (Lond.), 176, 1032-1033.

HILLARP, N - Å. (1958). Isolation and some biochemical properties of the catecholamine granules in the cow adrenal medulla. Acta physiol. scand., 43, 82-96.

HOFFMAN, F., HOFFMAN, E.J., MIDDLETON, S. and TALESNIK, J. (1945). The stimulating effect of acetylcholine on the mammalian heart and the liberation of an epinephrine-like substance by the isolated heart. Amer. J. Physiol., 144, 189-198.

HUCKOVIC, S. and MUSCHOLL, E. (1962). Die Noradrenalin Abgabe aus dem isolierten Kaninchenherzen bei sympathischer Nervenreizung und ihre pharmakologische Beeinflussung. Arch. Exp. Path. Pharmakol., 244, 81-96.

IVERSEN, L.L. and WHITBY, L.G. (1963). The subcellular distribution of catecholamines in normal and tyramine depleted mouse hearts. Biochem. Pharmacol., 12, 582-584.

IVERSEN, L.L. (1965a). The uptake of catecholamines at high perfusion concentrations in the rat isolated heart. A novel catecholamine uptake process. Brit. J. Pharmacol., 25, 18-33.

IVERSEN, L.L. (1965b). Inhibition of noradrenaline uptake by drugs. J. Pharm. Pharmacol., 17, 62-64.

IVERSEN, L.L., GLOWINSKI, J. and AXELROD, J. (1966). The Physiologic disposition and metabolism of norepinephrine in immunosympathectomized animals. J. Pharmacol. exp. Ther., 151, 2, 273-284.

KIRPEKAR, S.M. and MISU, Y. (1966). Dependence of norepinephrine release from spleen on Ca^{++}. Fed. Proc., 25, 384.

KOPIN, I.J., HERTTING, G. and GORDON, E.K. (1962). Fate of norepinephrine - H^3 in the isolated perfused rat heart. J. Pharmacol. exp. Ther., 138, 34-40.

LEVER, J.D. (1955). Electron microscopic observations on the normal and denervated adrenal medulla of the rat. Endocrinology, 57, 621-635.

LÖEWI, O. (1921). Ueber humorale Uebertragbarkeit der Herznervenwirkung. Arch. ges. Physiol., 189, 239-242.

LÜTTGAU, H.C. and NIEDERGERKE, R. (1958). The antagonism between calcium and sodium ions in the frog's heart. J. Physiol., 143, 486-505.

MINES, G.R. (1913). On functional analysis by the action of electrolytes. J. Physiol., 46, 188-235.

NAYLER, W.G. and EMERY, P.F. (1962). Effect of strontium on cardiac contractility and membrane resting potentials. Amer. J. Physiol., 203, 844-852.

PHILIPPU, A. and SCHÜMANN, H.J. (1962). Der Einfluss von Calcium auf die Brenzcatechinaminefreisetzung. Experientia, 18, 138-140.

POTTER, L.T. and AXELROD, J. (1962). Intracellular localization of catecholamines in the tissues of the rat. Nature, (Lond.), 194, 581-582.

POTTER, L.T. and AXELROD, J. (1963a). Properties of norepinephrine storage particles of the rat heart. J. Pharmacol. exp. Ther., 142, 299-305.

POTTER, L.T. and AXELROD, J. (1963). Subcellular localization of catecholamines in tissues of the rat. J. Pharmacol. exp. Ther., 142, 291-298.

POTTER, L.T. (1966). Storage of norepinephrine in sympathetic nerves. In Second Symposium on Catecholamines. Held in Milan 1965, ed. Acheson, G.H. Pharmacol. Revs., 18, 1, 439-451.

RICHARDSON, K.C. (1962). The fine structure of autonomic nerve endings in smooth muscle of the rat vas deferens. J. Anat., 96, 427-442.

RICHARDSON, J.A. and WOODS, E.F. (1959). Release of norepinephrine from the isolated heart. Proc. Soc. exp. Biol. Med. 100, 149-151.

RINGER, S. and SAINSBURY, H. (1883). An investigation regarding the action of strontium and barium salts compared with the action of lime on the ventricle of the frog's heart. The Practitioner, 31, 81-93.

ROSELL, S., KOPIN, I.J. and AXELROD, J. (1963). Fate of ^{3}H-noradrenaline in skeletal muscle before and following sympathetic stimulation. Amer. J. Physiol., 205, 317-321.

SCHMITERLÖW, C.G. (1948). The nature and occurrence of pressor and depressor substances in extracts from blood vessels. Acta physiol. scand., 16, Suppl. 56.

SCHÜMANN, H.J., SCHNELL, K. and PHILIPPU, A. (1964). Subcelluläre verteilung von Noradrenalin und Adrenalin im Meerschweinchenherzen. Arch. Exp. Path. Pharmak., 249, 251-266.

SHANES, A.M. (1958). Electrochemical aspects of physiological and pharmacological action in excitable cells. Pharmacol. Revs., 10, 59-273.

STJÄRNE, L. (1964). Studies of catecholamine uptake, storage and release mechanisms. Acta physiol. scand., 62, Suppl. 228.

STJÄRNE, L. (1966). Storage particles in noradrenergic tissues. In Second Symposium on catecholamines. Held in Milan, 1965, ed. Acheson, G.H., Pharmacol. Revs., 18, 1, 425-432.

STOLZ, F. (1904). Ueber Adrenalin und Alkylaminoacetobrenzcatechin. Ber. deutsch, chem. Gesellsch, 37, 4149-4154.

TAKESHIGE, C. and VOLLE, R.L. (1964). The Effects of Barium and other inorganic cations on sympathetic ganglia. J. Pharmacol. Exp. Ther., 143, 3, 327-334.

TEITEL, A. (1961). Cholinergic activity of Hyoscyamine. Nature, (Lond.), 190, 814.

TITUS, E. and DENGLER, H.J. (1966). The Mechanism of Uptake of norepinephrine. 2nd Symposium on Catecholamines, ed. Acheson, G.H., Pharmacol. Revs., 18, 525-535.

TITUS, E.O., MATUSSEK, N., SPIEGEL, H.E. and BRODIE, B.B. (1966). The effects of desmethylimipramine on uptake of dl-norepinephrine-7-H^3 in the heart. J. Pharmacol. exp. Ther., 152, 3, 469-477.

VOGT, M. (1959). The secretion of the denervated adrenal medulla of the cat. Brit. J. Pharmacol., 7, 325-330.

VULPIAN, A. (1856). Note sur quelques réactions propres à la substance des capsules surrénales. C.R. Acad. Sci. Paris, 43, 663.

WEINER, N. and JARDETZKY, O. (1964). A study of catecholamine nucleotide complexes by nuclear magnetic resonance spectroscopy. Arch. exp. Path. Pharmakol., 248, 308-318.

WIEDMANN, S. (1955). Effects of calcium ions and local anesthetics on electrical properties of Purkinje fibres. J. Physiol. 129, 568-582.

WOLFE, D.E. and POTTER, L.T. (1963). Localization of norepinephine in the atrial myocardium. Anat. Record., 145, 301.

APPENDIX I

Preparation of Solutions

All perfusion media were prepared in distilled water which had been passed through a deionizer.

Normal Krebs-bicarbonate Solution

The following mixture of salts was dissolved in water to produce a total volume of 1 litre:

Sodium Chloride (NaCl)	82.60 grams
Potassium Chloride (KCl)	4.22 "
Calcium Chloride ($CaCl_2$)	3.36 "
Potassium Phosphate (KH_2PO_4)	1.94 "
Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$)	3.50 "

Normal Krebs-bicarbonate solution was prepared by mixing 100 ml of the concentrated solution, 900 ml of distilled water, 2 grams of glucose and 5 grams of sodium bicarbonate contained in 192 ml of distilled water. The pH of this solution was 7.6. When aerated with 5% CO_2 and 95% O_2, the pH was 7.35 to 7.40. This solution contains the following millimolar concentrations:

NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM,
KH_2PO_4 1.2 mM, $MgSO_4.7H_2O$ 1.2 mM,
$NaHCO_3$ 25 mM, glucose 10 mM.

An acid Krebs medium was prepared by mixing 100 ml of the concentrated salt solution with 900 ml of distilled water (pH 5.5).

Preparation of dl-^{3}H-Noradrenaline for Infusion

The tritiated noradrenaline contained 43 μg noradrenaline per ml (S.A. 4.27 C/mM). A 1:100 dilution in 0.01N HCl of this solution contained 0.43 ug/ml. 7.5 ml of this solution was diluted to 40 ml with acid Krebs medium (Krebs medium containing no glucose or bicarbonate). The concentration of this solution was 80 ng noradrenaline/ml.

The infusion rate of tritiated amine was 0.22 ml/min. and this was further diluted by the perfusion medium entering at 3 ml/min. The final concentration of noradrenaline perfusing the heart was 5.5 ng/ml/min.

Acidified Salt-Saturated n-Butanol

20 grams of NaCl and 1 ml of concentrated HCl was added to 500 ml n-butanol and shaken for 1 hour.

Scintillation Fluid

The scintillation fluid was prepared by dissolving 100 grams naphthalene, 4.0 grams PPO (2,5 - Diphenyloxazole) and 0.1 grams POPOP (1,4 - Di - [2 - (5 - phenyloxazolyl)] benzene) in 1 litre of dioxane.

Modified Perfusion Media

(a) Calcium Free Krebs-bicarbonate medium

This medium was prepared by the omission of calcium chloride from a normal Krebs-bicarbonate perfusion medium. Strontium and barium substituted Krebs media were prepared by the addition of either 2.5 mM or 5 mM strontium chloride or barium chloride to a calcium free Krebs-bicarbonate solution as the experimental schedule demanded.

(b) High Potassium Krebs medium

A medium containing 59 mM potassium was prepared by the replacement of an equivalent amount of sodium chloride with potassium chloride. This concentration of potassium (59 mM) was maintained in calcium free media and in strontium substituted calcium free media.

(c) Low Sodium Krebs

236 mM sucrose was added to replace 118 mM NaCl. This solution contained 25 mM $NaHCO_3$.

(d) Excess Magnesium Krebs

In order to maintain tonicity when excess magnesium was employed, a sodium free medium was prepared and 80 mM magnesium chloride added.

APPENDIX II

TABLE 1

Normal Krebs Perfusion

Sample Calculation

Perfusion Procedure	Heart # 1 cpm released	Heart # 2 cpm released	Average cpm released	Average count x aliquot factor (10.2)	Efflux % of Total cpm associated with hearts
	2,611	3,173	2,892	29,498	40.15
	448	446	447	4,559	6.21
	212	255	233	2,377	3.24
	168	142	155	1,581	2.15
	131	96	114	1,163	1.58
	107	102	105	1,071	1.46
Efflux of ^{3}H-NA during perfusion with normal Krebs	97	85	82	928	1.26
	81	70	76	775	1.06
	70	67	69	704	0.96
	56	55	56	571	0.72
	63	71	67	683	0.93
	40	47	44	449	0.61
	50	48	49	500	0.68
	47	49	48	490	0.66
	31	42	37	377	0.51
	43	46	45	459	0.62
	28	50	39	398	0.54
	30	34	32	326	0.44
	37	33	35	357	0.49
	34	28	31	316	0.43
	39	25	32	326	0.44
	35	37	36	367	0.49
	23	38	31	316	0.43
	18	35	27	275	0.37
	16	24	20	204	0.27
	18	22	20	204	0.27

Average Total cpm released after infusion	=	50,204
Average cpm left in heart at end of experiment	=	23,250
Total cpm associated with hearts		73,454

Data plotted in Fig. 2

TABLE 2

Comparison of Normal Krebs, Calcium free Krebs and Calcium free Krebs + 2 mM Na_2EDTA

Perfusion Procedure	% Total cpm released A n = 4	% Total cpm released B n = 4	% Total cpm released C n = 2
Hearts A Normal Krebs	28.88	24.79	24.31
	4.50	4.34	8.23
	2.69	3.45	4.52
	1.96	2.75	3.20
	1.09	2.54	2.74
	1.15	1.97	2.27
	0.93	1.55	2.28
	0.84	1.24	2.01
	0.71	1.32	1.95
	0.68	0.95	1.98
Hearts B Calcium free Krebs + 0.2 mM Na_2EDTA	0.63	0.95	2.11
	0.58	0.95	1.89
	0.56	0.76	1.88
	0.52	0.72	1.71
	0.46	0.67	1.88
	0.52	0.69	1.76
	0.65	0.61	1.58
	0.66	0.51	1.47
	0.55	0.60	1.33
	0.55	0.54	1.37
	0.42	0.55	1.35
	0.34	0.47	1.15
	0.39	0.49	1.21
Hearts C Calcium free Krebs + 2 mM Na_2EDTA	0.51	0.43	1.09
	0.34	0.46	0.86

Data plotted in Fig. 3

TABLE 3

Replacement of Calcium free Krebs alone after perfusion with Calcium free Krebs + 2 mM Na_2EDTA

Perfusion Procedure	% Total cpm released A n = 2	% Total cpm released B n = 2
Hearts A & B Normal Krebs	31.6	31.0
	6.03	5.61
	3.39	3.36
	2.27	2.37
	1.72	1.89
	1.44	1.44
	1.22	1.15
	.903	1.02
	.860	.84
	.735	.75
Hearts A & B Calcium free Krebs + 2 mM Na_2EDTA	.948	1.25
	2.15	2.54
	1.77	2.04
	1.49	1.85
	1.45	1.70
	1.46	1.60
	1.46	1.70
	1.57	1.60
Hearts A Calcium free Krebs + 2 mM Na_2EDTA	1.64	0.94
	1.77	0.81
	1.74	0.76
Hearts B Calcium free Krebs without Na_2EDTA	1.79	0.71
	1.80	0.51
	1.77	0.61
	1.90	0.49
	2.04	0.51

Data plotted in Fig. 4

TABLE 4

Calcium free + 2 mM Na_2EDTA and Normal Krebs Crossover Experiments

Perfusion Procedure	% Total cpm released A n = 3	% Total cpm released B n = 1	Perfusion Procedure	% Total cpm released C n = 2	% Total cpm released D n = 2
Hearts A & B Calcium free Krebs + 2 mM Na_2EDTA	38.2	35.4	Hearts C & D Normal Krebs	15.75	18.41
	8.59	7.68		3.99	5.93
	6.56	6.35		2.55	3.78
	4.01	4.14		1.81	2.12
	3.44	3.06		1.31	1.46
	3.12	2.90		1.03	1.03
	2.85	2.66		0.79	1.14
	2.49	2.43		0.72	0.88
	2.84	2.13		0.67	0.79
	2.71	2.12		0.59	0.76
Hearts A Normal Krebs	2.13	1.83	Hearts C Calcium free Krebs + 2 mM Na_2EDTA	0.57	0.61
	1.69	1.95		2.23	0.50
Hearts B Calcium free Krebs + 2 mM Na_2EDTA	1.14	1.74		2.24	0.61
	0.9	1.76		2.05	0.56
	0.63	1.64		1.92	0.60
	0.52	1.64	Hearts D Normal Krebs	1.72	0.61
	0.48	1.54		1.89	0.70
	0.45	1.26		1.97	0.64
Hearts A & B Normal Krebs	0.40	1.41	Hearts C & D Normal Krebs	2.42	0.62
	0.41	1.48		2.45	0.55
	0.51	1.45		1.79	0.44
	0.70	1.35		1.32	0.47
	0.85	1.06		1.40	0.60
	1.08	0.94		1.24	0.60
	1.21	0.99		1.16	0.58
	1.23	0.94		1.10	0.58

Data plotted in Fig. 5

TABLE 5

Extended Crossover Experiments involving Calcium free, Calcium free + 2 mM Na_2EDTA and Normal Krebs

Perfusion Procedure	% Total cpm released A n = 2	% Total cpm released B n = 2
Hearts A Normal Krebs	27.12	29.97
	4.805	7.16
Hearts B Calcium free Krebs	2.52	4.23
	1.68	2.63
	1.54	1.93
	1.23	1.59
	1.16	1.53
	1.01	1.39
	0.81	1.33
	0.75	1.23
Hearts A Calcium free Krebs	1.02	1.04
	1.87	0.93
Hearts B Normal Krebs	1.53	0.92
	1.33	1.03
	1.11	0.91
Hearts A Normal Krebs	0.94	0.94
	0.61	0.75
Hearts B Calcium free Krebs	0.65	0.69
	0.66	0.78
	0.47	0.65
Hearts A Normal Krebs	0.53	0.76
	0.44	1.15
Hearts B Calcium free Krebs +	0.54	1.44
2 mM Na_2EDTA	0.35	1.37
	0.36	1.49
Hearts A Calcium free Krebs +	1.02	1.69
2 mM Na_2EDTA	1.50	1.05
	6.65	0.81
Hearts B Normal Krebs	0.99	0.53
	1.82	0.58

Data plotted in Fig. 6

TABLE 6

Replacement of Calcium with Strontium

Perfusion Procedure	% Total cpm released A n = 3	% Total cpm released B n = 1
Hearts A & B Calcium free Krebs + 2 mM Na_2EDTA	45.20	48.54
	14.15	12.06
	7.07	6.01
	4.41	3.38
	3.55	3.02
	2.72	2.42
	2.35	2.39
	1.81	2.10
	1.48	1.35
	1.22	1.27
Hearts A 2.5 mM Strontium (Normal)	1.27	0.89
	0.98	0.97
Hearts B Calcium free Krebs + 2 mM Na_2EDTA	0.80	0.93
	0.63	0.80
	0.42	0.61
	0.37	0.55
	0.35	0.80
	0.30	0.73
Hearts A & B Calcium free Krebs + 2 mM Na_2EDTA	0.33	1.00
	0.30	1.08
	0.33	1.00
	0.41	0.97
	0.38	0.93
	0.41	0.87
	0.55	0.94
	0.60	0.64

Data plotted in Fig. 7

TABLE 7

Effect of Replacing Calcium (2.5 mM) with Barium (2.5 mM)

Perfusion Procedure	% Total cpm released A n = 6	% Total cpm released B n = 2
Hearts A & B Normal Krebs	25.19	31.18
	6.65	5.29
	4.17	2.47
	2.27	1.97
	2.18	1.38
	1.67	1.36
	1.34	1.21
	1.02	0.75
	0.96	0.85
	0.69	0.75
Hearts A Calcium free Krebs containing 2.5 mM Barium	0.67	0.69
	0.89	0.55
	1.43	0.56
Hearts B Normal Krebs	1.37	0.50
	0.93	0.43
	0.86	0.41
	0.76	0.42
	0.73	0.42
Hearts A & B Normal Krebs	0.67	0.45
	0.66	0.29
	0.73	0.43
	0.75	0.27
	0.73	0.40
	0.73	0.33
	0.65	0.26
	0.71	0.28

Data plotted in Fig. 8

TABLE 8

Effect of Barium (5.0 mM) added to Calcium free Krebs

Perfusion Procedure	% Total cpm released A n = 3	% Total cpm released B n = 1
Hearts A & B Normal Krebs	39.65	36.11
	7.55	5.76
	4.02	3.28
	2.40	2.19
	1.75	2.19
	1.53	1.20
	1.17	1.28
	1.10	0.73
	0.81	0.82
	0.80	0.75
Hearts A Calcium free Krebs containing 5.0 mM Barium	0.75	0.64
	4.38	0.53
	3.02	0.53
Hearts B Normal Krebs	2.42	0.56
	2.24	0.57
	1.93	0.64
	1.83	0.48
	1.45	0.54
Hearts A & B Normal Krebs	1.27	0.41
	0.99	0.50
	0.65	0.46
	0.33	0.41
	0.35	0.47
	0.32	0.45
	0.28	0.48
	0.25	0.27

Data plotted in Fig. 9

TABLE 9

Potassium stimulated Release during perfusion with Normal and Calcium free Krebs

Perfusion Procedure	% Total cpm released A n = 4	% Total cpm released B n = 2
Hearts A Normal Krebs	26.98	38.23
	4.65	5.92
Hearts B Calcium free Krebs	2.61	4.16
	1.79	2.80
	1.49	2.27
	1.21	1.69
	0.96	1.55
	0.74	1.21
	0.64	1.14
	0.51	0.95
Hearts A 59 mM Potassium Krebs	0.62	0.86
	1.17	0.74
Hearts B 59 mM Potassium in Calcium free Krebs	0.94	0.70
	0.915	0.83
	0.66	0.79
	0.61	0.69
	0.48	0.61
	0.55	0.63
Hearts A Normal Krebs	0.44	0.59
	0.44	0.59
Hearts B Calcium free Krebs	0.34	0.57
	0.28	0.48
	0.27	0.47
	0.32	0.45
	0.25	0.47
	0.26	0.37

Data plotted in Fig. 10

TABLE 10

Strontium substituted Krebs and stimulation by Potassium

Perfusion Procedure	% Total cpm released A n = 2	% Total cpm released B n = 2
Hearts A Calcium free Krebs	38.69	38.94
	7.87	6.24
Hearts B Strontium (2.5 mM) substituted Calcium free Krebs	4.09	3.33
	2.90	2.01
	2.13	1.64
	1.73	1.38
	1.47	0.96
	1.25	0.67
	1.09	0.67
	0.79	0.63
Hearts A 59 mM Potassium in Calcium free Krebs	0.79	0.91
	0.72	2.86
	0.62	1.45
Hearts B 59 mM Potassium in Strontium (2.5 mM) substituted Calcium free Krebs	0.55	1.21
	0.78	1.13
	0.38	0.99
	0.39	0.76
	0.43	0.61
Hearts A Calcium free Krebs	0.38	0.70
	0.31	0.61
Hearts B Strontium (2.5 mM) substituted Calcium free Krebs	0.32	0.59
	0.31	0.46
	0.25	0.40
	0.33	0.48
	0.27	0.48
	0.26	0.48

Data plotted in Fig. 11

TABLE 11

Perfusion with Calcium free Krebs and the stimulating action of Noradrenaline

Perfusion Procedure	% Total cpm released A n = 6	% Total cpm released B n = 4
Hearts A Normal Krebs	21.88	33.91
	4.88	4.13
Hearts B Calcium free Krebs	3.14	2.56
	2.12	2.07
	1.59	1.40
	1.39	1.18
	1.18	1.15
	0.89	0.99
	0.77	0.83
	0.66	0.87
Hearts A & B 1 μM Noradrenaline	1.27	1.22
	2.37	1.31
	1.85	1.01
	1.36	0.99
	1.16	0.87
	0.84	0.66
	0.74	0.69
	0.61	0.61
Hearts A & B 2 μM Noradrenaline	0.60	0.93
	2.22	1.02
	1.77	0.97
	1.39	0.90
	1.13	0.63
	0.89	0.71
	0.75	0.49
	0.68	0.58

Data plotted in Fig. 12

TABLE 12

Perfusion with Calcium free Krebs + 2 mM Na_2EDTA and the stimulating action of Noradrenaline

Perfusion Procedure	% Total cpm released A n = 4	% Total cpm released B n = 4
Hearts A Normal Krebs	26.49	24.31
	4.33	8.23
Hearts B Calcium free Krebs + 2 mM Na_2EDTA	2.69	4.52
	2.10	3.20
	1.78	2.74
	1.42	2.27
	1.14	2.28
	1.15	2.01
	1.06	1.95
	0.74	1.95
Hearts A & B 1 μM Noradrenaline	1.40	1.98
	2.05	2.11
	1.70	1.89
	1.30	1.88
	0.98	1.71
	0.74	1.88
	0.63	1.76
	0.54	1.58
Hearts A & B 2 μM Noradrenaline	1.34	1.47
	2.11	1.33
	1.79	1.37
	1.19	1.35
	0.98	1.15
	0.80	1.22
	0.69	1.09
	0.60	0.86

Data plotted in Fig. 13

TABLE 13

Strontium substituted Krebs and Noradrenaline stimulation

Perfusion Procedure	% Total cpm released A n = 2	% Total cpm released B n = 2
Hearts A Calcium free Krebs	51.54	41.67
	7.69	7.16
Hearts B Strontium (2.5 mM) substituted Calcium free Krebs	4.39	3.99
	2.46	2.48
	2.02	3.62
	1.63	1.90
	1.52	1.61
	1.14	1.31
	1.17	1.24
	0.86	1.13
Hearts A & B 1 µM Noradrenaline	1.08	0.94
	0.93	1.36
	1.07	1.59
	0.84	1.01
	0.85	0.88
	0.78	0.84
	0.67	0.77
	0.69	0.81
Hearts A & B 2 µM Noradrenaline	0.77	1.13
	0.75	1.59
	0.80	1.49
	0.88	0.83
	0.75	0.67
	0.42	0.64
	0.41	0.62
	0.49	0.54

Data plotted in Fig. 14

TABLE 14

Perfusion with low Sodium Krebs

Perfusion Procedure	% Total cpm released A n = 3	% Total cpm released B n = 1
Hearts A & B Normal Krebs	25.57	42.45
	6.09	5.77
	3.75	2.55
	2.37	1.76
	1.81	1.23
	1.48	1.16
	1.04	0.88
	0.92	0.72
	0.76	0.70
	0.63	0.50
Hearts A low Sodium Krebs	0.50	0.43
	0.50	0.33
Hearts B Normal Krebs	0.54	0.23
	0.62	0.25
	0.65	0.27
	0.67	0.29
	0.71	0.34
	0.88	0.38
Hearts A & B Normal Krebs	0.85	0.28
	0.56	0.27
	0.47	0.29
	0.38	0.28
	0.54	0.27
	0.56	0.29
	0.42	0.22
	0.49	0.33

Data plotted in Fig. 15

TABLE 15

Effect of Krebs containing 80 mM Magnesium

Perfusion Procedure	% Total cpm released A n = 4	% Total cpm released B n = 4
Hearts A & B Normal Krebs	33.07	30.72
	4.65	6.53
	2.82	2.90
	1.93	2.01
	1.40	1.53
	1.15	1.10
	1.03	1.10
	0.85	0.90
	0.73	0.86
	0.62	0.70
Hearts A Krebs containing 80 mM Magnesium	0.73	0.72
	1.32	0.67
Hearts B Normal Krebs	1.23	0.64
	1.02	0.59
	1.13	0.57
	1.03	0.53
	0.91	0.60
	0.86	0.55
Hearts A & B Normal Krebs	0.81	0.50
	0.59	0.50
	0.54	0.43
	0.69	0.41
	0.70	0.43
	0.73	0.46
	0.71	0.41
	0.73	0.42

Data plotted in Fig. 16

TABLE 16

Effects of Acetylcholine Perfusion

Perfusion Procedure	% Total cpm released A n = 2	% Total cpm released B n = 2
Hearts A Normal Krebs +	24.4	16.8
10 µg/ml Atropine	4.09	5.23
	2.24	3.11
Hearts B Normal Krebs	1.59	2.06
	1.58	1.44
	1.47	1.31
	1.38	1.18
	1.09	0.85
	.97	0.83
	0.75	0.83
Hearts A & B 50 µg/ml Acetylcholine	0.74	0.57
	0.63	0.57
	0.77	0.70
	0.63	0.77
	0.66	0.63
	0.57	0.49
	0.50	0.45
	0.43	0.35
Hearts A & B 100 µg/ml Acetylcholine	0.34	0.36
	0.37	0.43
	0.46	0.57
	0.39	0.55
	0.30	0.55
	0.36	0.45
	0.43	0.41
	0.43	0.42

Data plotted in Fig. 17

B29866